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ADVANCED COMPUTATIONAL METHODOLOGIES IN TOMOGRAPHIC PHASE IMAGING FLOW CYTOMETRY

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Есть ещё порох в
пороховницах!

- Н.В. Гоголь,
Тарас Бульба

*Non chi comincia,
ma quel che persevera*

ADVANCED COMPUTATIONAL METHODOLOGIES IN TOMOGRAPHIC PHASE IMAGING FLOW CYTOMETRY

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for the fulfillment of the Degree of Doctor of Philosophy
in Information and Communication Technology for Health
by **FRANCESCA BORRELLI**

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Candidate's declaration

I hereby declare that this thesis submitted to obtain the academic degree of Philosophiæ Doctor (Ph.D.) in Information and Communication Technology for Health is my own unaided work, that I have not used other than the sources indicated, and that all direct and indirect sources are acknowledged as references.

Parts of this dissertation have been published in international journals and/or conference articles (see list of the author's publications at the end of the thesis).

Napoli, October 31, 2023



Francesca Borrelli

Abstract

Single-cell analysis will constitute the future of precision medicine and early diagnosis. To date, the gold-standard cytometric technique is Fluorescence Imaging Flow Cytometry (FIFC), which allows to record 2D images of stained single cells as they flow through a measuring device, granting an elevate throughput. Since FIFC can provide large, informative image datasets, the combination with Artificial Intelligence (AI) - based analysis tools can allow a fast, automatic, and objective cell phenotyping. However, the staining process can affect in different ways the cells under investigation. In fact, contrast agents can disturb the normal function of living cells (cytotoxicity) and lasers used in FIFC can be toxic for them as well (phototoxicity). Conversely, label-free optical imaging techniques avoid cells staining, thus becoming the most promising solution to overcome the intrinsic limitations of the FIFC. Among label-free microscopies, Tomographic Phase Microscopy (TPM) represents the highest informative imaging strategy as it allows to reconstruct the volumetric distribution of the Refractive Index (RI) at the single-cell level. The cellular RI is a key biophysical parameter proven to be an effective descriptor of cellular heterogeneity and physiopathology. For the first time in 2017, TPM has been demonstrated in Flow Cytometry (FC) mode, paving the way to the Tomographic Phase Imaging Flow Cytometry (TPIFC) technology. In TPIFC, digital holograms of single cells are recorded in continuous flow while the samples rotate in microfluidic environment. As it allows to conjugate the high throughput of FC technology and the availability of the full 3D RI information, the TPIFC tool is expected to create a breakthrough in the cell biology studies and in the clinical practice. Nowadays, the research and commercial trend is to combine TPIFC technology with Lab on Chip (LOC) devices to go towards telehealth applications. The desired LOC devices will be compact, cheap and charac-

terised by inexpensive components and acceptable computational effort. This creates a challenge for [TPIFC](#), going towards the paradigm of Computational Tomography, according to which the complexity will be shifted from the optical and hardware point of view to the numerical one. Accordingly, advanced computational methodologies are needed to achieve the above goal. In addition, to keep the elevate throughput of [FC](#), fast tomographic approaches are sought after, able to reconstruct with a reduced number of acquisitions. Therefore, several computational strategies are developed in this Ph.D. Thesis for transferring the original proof of concept of [TPIFC](#) into a concrete, [LOC](#)-oriented technology for the single-cell analysis. In particular, powerful numerical approaches to reconstruct with reduced number of measurements and with low inference time are developed. Moreover, [AI](#) models for phenotyping cancer cells are discussed. Finally, a preliminary theoretical work is conducted for the higher-order description of the scattering process, to improve the tomographic imaging description and inversion approaches. In the near future, the attained results are expected to contribute in providing a solution to the challenging topic of the label-free single-cell analysis, for example focusing on early diagnosis of cancer and the development of personalised therapies by means of blood tests.

Keywords: Digital Holography, Tomographic Phase Microscopy, Artificial Intelligence, Single-cell Analysis, Imaging Flow Cytometry, High Order Scattering Modelling.

Sintesi in lingua italiana

L'analisi al livello di singola cellula costituirà il futuro della medicina di precisione e della diagnosi precoce. Ad oggi, la tecnica di riferimento per l'analisi cellulare è la citometria a flusso basata su fluorescenza (FIFC), che consente di registrare immagini 2D di singole cellule taggate da agenti marcatori mentre scorrono attraverso un dispositivo di misura, consentendo così un throughput elevato. Data la possibilità offerta dalla FIFC di ottenere dataset grandi ed informativi in poco tempo, la combinazione di questa con tecniche di analisi basate su AI permettono inoltre una fenotipizzazione delle cellule in modo rapido, automatico ed oggettivo. Tuttavia, il processo di marcatura delle cellule può portare alla loro alterazione distruttiva. Infatti, sia gli agenti di contrasto utilizzati che i laser impiegati nell'indagine possono condizionare lo stato dei campioni, i primi compromettendone le funzionalità fisiologiche (citotossicità), i secondi essendo spesso tossici per loro (phototoxicity). Diversamente, le tecniche di imaging ottico label-free consentono di evitare la marcatura dei campioni, divenendo così l'alternativa più promettente per superare le limitazioni legate all' FIFC. Tra le varie tecniche di microscopia label-free esistenti, la TPM rappresenta la strategia di imaging più informativa, consentendo di ricostruire l'intero profilo volumetrico di RI al livello di singola cellula. Infatti, la distribuzione di RI di un campione rappresenta un parametro biofisico cruciale, essendo stato dimostrato essere un descrittore della eterogeneità cellulare e della fisiopatologia. Per la prima volta nel 2017 la tecnologia TPM è stata dimostrata in flusso, aprendo la strada alla cosiddetta TPIFC. In questa tecnologia, gli ologrammi digitali relativi alle singole cellule sono registrati in flusso continuo mentre i campioni scorrono e ruotano in un sistema microfluidico. Permettendo di coniugare l'elevato throughput della FC e la disponibilità dell'intera informazione 3D sull' RI, la TPIFC è attesa essere come la tecnologia capace di creare una

svolta nello studio biologico delle cellule e nella pratica clinica.

Ad oggi, il trend sia di ricerca che commerciale prevede di combinare la **TPIFC** con il mondo dei **LOC**, con l'idea di andare verso applicazioni di telehealth. I dispositivi **LOC** desiderati sono compatti, economici e caratterizzati da componentistica economica e capacità computazionali ridotte. Ciò crea una sfida nell'ambito della **TPIFC**, andando incontro al cosiddetto paradigma della Tomografia Computazionale, secondo il quale la complessità della tecnologia sarà spostata dal punto di vista ottico ed hardware a quello numerico. In quest'ottica, metodologie computazionali avanzate sono necessarie per ottenere le performance desiderate, laddove la parte hardware sarà più carente. Inoltre, per mantenere il vantaggio del throughput elevato apportato dalla **FC**, approcci di ricostruzione tomografica veloci sono desiderati, che consentano di ricostruire i campioni a partire da un numero di misure ridotto. Pertanto, in questa tesi di dottorato diverse strategie numeriche sono sviluppate e proposte, con l'idea di trasferire la proof of concept della **TPIFC** verso una tecnologia concreta e orientata al **LOC** per l'analisi a singola cellula. In particolare, sono sviluppati e discussi potenti approcci numerici che consentano di ricostruire utilizzando poche proiezioni in ingresso e con un tempo di inferenza basso. In aggiunta, modelli basati su **AI** per la fenotipizzazione di cellule tumorali sono introdotti. Infine, un lavoro teorico preliminare è condotto per un modelling di ordine superiore del processo di scattering, per migliorare sia la descrizione dell'imaging tomografico che di conseguenza gli approcci di inversione. Nel prossimo futuro, i risultati discussi in questa tesi sono attesi essere un contributo nel trovare una soluzione al problema della analisi label-free a singola cellula, con applicazioni importanti come la diagnosi precoce di cancro e lo sviluppo di terapie personalizzate basate sull'analisi del sangue.

Olografia Digitale, Tomografia microscopica di fase, Intelligenza Artificiale, Analisi a singola cellula, Imaging Flow cytom-

etry, Modelli di scattering di ordine superiore

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List of Acronyms

The following acronyms are used throughout the thesis.

AI	Artificial Intelligence
ADM	Alternating Direction Method
ALM	Augmented Lagrangian Method
BN	Batch Normalization
BSP	Beam Splitter
BS	Batch Size
CAN	Context Aggregation Network
CNN	Convolutional Neural Network
CTC	Circulating Tumour Cells
CT	Computed Tomography
DCNN	Deep Convolutional Neural Network
DH	Digital Holography
DHM	Digital Holographic Microscopy

DL	Deep Learning
DM	Dry Mass
FACS	Fluorescence Activated Cell Sorting
FBP	Filtered Back-Projection
FBPR	Filtered Back-Propagation
FC	Flow Cytometry
FDT	Fourier Diffraction Theorem
FIFC	Fluorescence Imaging Flow Cytometry
FM	Fluorescence Microscopy
FOV	Field of View
FST	Fourier Slice Theorem
GAN	Generative Adversarial Network
HO	High Order
HOTV	High Order Total Variation
HR	High Resolution
IFC	Imaging Flow Cytometry
ISC	Illumination Scanning Configuration
LAT	Limited Angle Tomography
LB	Liquid Biopsy
LOC	Lab on Chip

LR	Learning Rate
LRE	Low Resolution
MAE	Mean Absolute Error
MFSR	Multi-Frame Image Super Resolution
ML	Machine Learning
MO	Microscope Objective
MRI	Magnetic Resonance Imaging
MSE	Mean Standard Error
NA	Numerical Aperture
OC	Ovarian Cancer
ODT	Optical Diffraction Tomography
OPD	Optical Path Delay
PA	Polynomial Annihilation
PBS	Polarised Beam Splitter
PCA	Principal Components Analysis
PET	Positron Emission Tomography
PSF	Point Spread Function
PSNR	Peak Signal to Noise Ratio
QPI	Quantitative Phase Imaging
QPM	Quantitative Phase Map

RBC	Red Blood Cells
ReLU	Rectified Linear Unit
RI	Refractive Index
ROI	Region of Interest
SD	Shearing Device
SISR	Single Image Super Resolution
SR	Super Resolution
SRC	Sample Rotation Configuration
SSIM	Structural Similarity Index Matrix
TC	Tamura Coefficient
TPIFC	Tomographic Phase Imaging Flow Cytometry
TPM	Tomographic Phase Microscopy
TSI	Tamura Similarity Index
TV	Total Variation
T-SNE	t-Distributed Stochastic Neighbour Embedding
WBC	White Blood Cell

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Introduction

Nowadays, cellular inspection is an essential tool for precision medicine and early diagnosis. In fact, single-cell level measurements of morphological and biochemical features provide unique information allowing to identify pathological states or to detect rare cells with specific features (e.g., tumor cells, stem cells, etc.) at early stages. The gold standard cytological technique is [FC](#), a tool that allows to measure multiple cellular parameters at high throughput and to obtain statistical information about the population under exam. A milestone development of this technique consisted in the embedding of imaging into the [FC](#) paradigm, leading to Imaging Flow Cytometry ([IFC](#)) systems. Indeed, the availability of images for each cell allows to restore the single-cell level specificity, which is partially lost in [FC](#), and to integrate [AI](#) to process the data acquired.

Nowadays, one of the most promising developments of [IFC](#) technique consists in the use of label-free Quantitative Phase Imaging ([QPI](#)) methods as imaging methodics, providing quantitative measurements for the cells and overcoming the drawbacks linked to the use of chemical stains. Indeed, although capable of offering high-contrast imaging with intracellular specificity, chemical stains and dyes required by standard microscopy to image phase samples such as cells are often incompatible with live cell analysis. In addition to altering the behavior of the samples, the use of labels imposes a sample preparation stage which is costly and labor intensive. Moreover, photobleaching can alter the quality of the imaging, especially in long run experiments. Among other [QPI](#) techniques, Digital Holography ([DH](#)) rep-

resents a valuable choice for the possibility to operate a numerical post processing refocusing. The use of [DH](#) allows to overcome the use of external labels as, thanks to its interferometric principle, the entire complex field can be recorded. The acquired images, termed Quantitative Phase Map ([QPM](#))s, are integral measurements of a coupled information about [RI](#) and physical thickness of the sample. The whole 3D [RI](#) information can be retrieved exploiting the tool of computed tomography, leading to the so-called [TPM](#) technique. Proposed for the first time in 2006, [TPM](#) allows to extract the maximum information contained into the [QPM](#)s about the samples under examination by acquiring [QPM](#)s corresponding to different angular views and opportunely combining them. Different techniques have been implemented for [TPM](#), but the state-of-the-art methodologies mainly work in a static mode. According to this technique, [QPM](#)s relative to different orientations are acquired fixing the position of the sample and scanning the illumination used to probe it. This methodology not only lowers the throughput as the sample needs to be physically placed in an adequate position for the measurements, but also has the potentiality to alter it. The possibility to conjugate [TPM](#) informativeness with a high throughput is obtained with [TPIFC](#)s. This technology, demonstrated as a proof of concept for the first time at ISASI-CNR in 2017, allows to perform the tomographic reconstructions of samples acquired in an in-flow condition. Specifically, as the samples flow into a microfluidic device, thanks to hydrodynamic forces they are induced to rotate, while a standing camera records the [QPM](#)s relative to different orientations. This grants an isotropic examination of the samples with a minimal risk of altering them; in addition, cells are recorded in suspension in a buffer medium and not in adhesion spread over a support, allowing to reveal their 3D actual shape and inner organisation.

The modern trend in [TPIFC](#)s consists in the embedding of this technique at [LOC](#) scale, going towards telemedicine applications. At this purpose,

the desire is to develop always smaller, cheaper systems, suitable for use outside optical labs and provided with processing methods capable of providing the results in a short time. Therefore, the optical performance of the setups will be lowered as will the computational capabilities, reduced in [LOC](#) devices. In this context, a challenge is posed for the tomographic reconstruction as the complexity of the technique will be shifted from the optical point of view to a numerical one. This paradigm, termed here Computational Tomography, is the main focus of this thesis. In order to obtain [TPM](#) setups suitable to be used outside of optical labs, common path setups have been introduced to overcome the limitations intrinsically linked to the Mach-Zehnder architectures. Indeed, even though Mach-Zehnder devices are among the most used in [DH](#), they strongly suffer from environmental disturbances, differently from common path-based setups which are robust to this issue. At this purpose, at ISASI-CNR a new quasi-common path lateral-shearing holographic optical setup has been proposed for the first time for [TPIFC](#). In addition, it makes use of cheaper components, going towards a commercial-oriented system. As a result, the setup is characterised by a lowered resolution and smaller number of orientations collectable for each sample, enhancing the ill posedness nature of the tomographic problem. The standard tomographic reconstruction algorithms are not suitable for this case and different solutions have to be explored, often making use of iterative solvers. Among others, Total Variation ([TV](#))-based ones represent the state-of-the-art numerical iterative solution for the case of limited number of orientation-data and allow to obtain reconstructions of elevate quality. However, for the nature of the algorithm the reconstructions present artefacts mining their reliability for biological applications. High order solvers have been proposed in the literature to overcome the limitations associated to [TV](#), but have never been used in the context of [TPIFC](#). Here, for the first time this application is successfully discussed, providing a numerical technique based on the High Order Total Varia-

tion (HOTV) suitable for the reconstruction of the data acquired with the developed common path setup.

This approach, as will be shown, can successfully mitigate the limited number of orientation problem and produce reconstruction of optimal quality, superior to TV and the standard solvers. However, it comports severe drawbacks such as the high computational cost and the elevate reconstruction time, non compatible with the sought-after quasi-real time visualisation. A key to overcome this limitation lays in the use of Deep Learning (DL) techniques. Recently, these techniques have been successfully integrated into computed tomography, allowing to obtain accurate and fast tools capable of overcoming many bottlenecks of the procedure. In this thesis, with the idea of going towards quasi-real time reconstructions, a particularly lightweight Convolutional Neural Network (CNN) architecture is exploited, the CAN, and used in a tomographic inversion approach capable of producing reconstructions in approximately 30% of the time needed by the state-of-the-art DL-based solvers, and characterised by a reduced memory occupation. This represents a first step towards the LOC application of TPIFC. In addition, from the DL world solutions useful to restore the lost resolution due to the reduced optical performance of the systems can be integrated, and preliminary studies on this theme have been conducted in this thesis.

Finally, a different powerful integration of AI-based techniques regards their applications to 2D QPI as a tool for performing classification between QPMs, paving the way to the identification of Circulating Tumour Cells (CTC)s for Liquid Biopsy (LB) applications. Indeed, this technique is becoming an essential support to tissue biopsy in cancer detection, allowing early screening and therapeutic monitoring. Starting from single or multiple QPMs acquired of a single cell, the information there contained can be used to discriminate between normal samples or altered ones through Machine Learning (ML) or DL.

Concluding, concerning the tomographic reconstruction, other modellings are presented in this thesis. Indeed, the tomographic reconstruction is often associated to an underlying approximation of the scattering process, based on the first order Born or first order Rytov descriptions. These scattering descriptions are first order models and are the basis of all the numerical reconstruction approaches discussed in this thesis. Even though extremely used, these approximations are often not accurate enough for the modelling of the physical phenomena involved, leading to inaccurate solvers. Interesting alternatives can come from the employment of higher order modellings which might provide a more accurate description of the process. In particular, although the Rytov approximation is often used and recognised to be superior to the Born one in some applications, no systematic study has been conducted over a higher order Rytov modelling, conversely to higher order Born's. Here, a simple case is treated, discussing the second order Rytov approximation and developing its mathematical implementation for the case of the scattering of a 2D, homogeneous cylinder in section. This work paves the way to the application of this description to more complex real-life tomographic problems such as [TPM](#).

The thesis is structured as follows. In Chapter [1](#), an introduction over the [DH](#) technique is provided, with an insight of its application in biomedicine. Moreover, the state-of-the-art cytological techniques, [FC](#) and [IFCs](#), are described, together with the main research trends associated.

In Chapter [2](#), the computed tomography is introduced and the [TPM](#) and [TPIFC](#) techniques are discussed in detail. A description of the classical reconstruction algorithms for tomography is provided, together with the available state-of-the-art solvers.

In Chapter [3](#), the paradigm of Computational Tomography is introduced, and a new setup developed at ISASI-CNR with the idea of going towards portable applications is presented. In addition, a powerful solver based on [HOTV](#) is applied for the first time to [TPIFC](#) for the processing of

tomographic data, and the results obtained for experimental samples are illustrated, together with possible future developments based on fractional calculus.

Chapter 4 mainly focuses on the combination of AI techniques with the TPIFC technology. First, a simulated tomographic model is presented, developed as a tool used for the training of DL models. Then, a DL based tomographic reconstruction procedure is described for 2D data, where CNNs are trained to enhance the tomograms reconstructed with classical algorithms in the case of reduced number of orientations. In particular, a CNN allowing an extremely reduced inference time and low memory occupation is chosen for the purpose. The procedure is also adapted to the reconstruction of 3D tomographic data for inference for a realistic use-case. Finally, the open problem of resolution enhancement is introduced and discussed and some preliminary procedures are described to approach it with DL approaches. Also, AI is used for classification purposes detecting cells target from 2D QPI data. Via ML and DL, the 2D QPMs relative to healthy and cancer ovarian cells are classified. Moreover, to make this sorting task more performing and robust, CNNs working on multiple 2D QPMs are used. The advantage in this approach is that 3D information is included, embedded into multiple-views QPMs, but without resorting to tomographic techniques to explicitly resolve it, lowering the computational burden.

Finally, in Chapter 5 the preliminary work about the second order Rytov modelling is presented and applied for the first time to the case of the scattering from an 2D homogeneous cylinder. This seminar discussion paves the way to future applications in the field of tomographic reconstruction and TPIFC.

Quantitative Phase Imaging by Digital Holography

Imaging of biological cells and tissues is fundamental for biological research and medical diagnosis and is an extremely active research field. The fundamental principle used is microscopy, and the state-of-the-art cytological investigation technologies are FC and its more recent advancement IFC. However, label-free QPI techniques rose to interest as an alternative to standard imaging and quickly become a fundamental tool for cellular investigation. Incorporating DH as an imaging technique, the acquired images (QPMs) result integral measurements of the coupled information about the RI and physical thickness of the sample, providing a quantitative measurement without the need of exogenous straining.

In this Chapter, the general concepts about DH and QPI are introduced, along with an overview on their applications in biomedical investigation. The description of the FC and IFC techniques is provided, together with the applications of QPI in IFC. Finally, an insight over the integration of label-free IFC techniques into commercial LOC devices is given.

1.1 Introduction to quantitative phase microscopy

The gold standard technique for microscopic imaging is brightfield microscopy. Contrast is the quantity defining how the sample is distinguishable from the background [1]. In the case of brightfield microscopy, the

quantity determining the contrast is absorption; however, most biological samples show a low absorption property. For them, scattering determines the contrast, occurring as the incident light interacts with the internal RI distribution of the sample. For thin samples exhibiting little relative variation of RI, the scattering might be several orders of magnitude smaller than the incident light, making its contribution too faint to provide appreciable contrast [1]. In fact, these samples are referred to as phase objects, as their effect is to induce a phase delay into the incident light. The gold standard imaging technique used for these samples is Fluorescence Microscopy (FM), which consists in converting them into amplitude objects using various stains or fluorescent tags [2], successfully providing high contrast images with good internal specificity but leading to important drawbacks. Their use requires a sample preparation stage depending on skilled personnel. The effects of photobleaching and phototoxicity might interfere with the experiment itself. Furthermore, the fluorescence dies and tag might alter the normal physiology of the cell without the possibility to be re-injected into the human body or to reuse them for further investigation purposes [1]. All these drawbacks limit the application of such methods to live cell analysis. Additionally, they are qualitative methods, therefore, the acquired images do not convey further physical information. A powerful alternative for the imaging of phase-objects is the use of QPI methods. These methods offer the possibility to measure the Optical Path Delay (OPD) introduced by the sample:

$$\begin{cases} QPM(x, y) = \frac{2\pi}{\lambda} OPD(x, y) \\ OPD(x, y) = \int_z [n(x, y, z) - n_m] dz \end{cases} \quad (1.1)$$

where λ is the wavelength, z is the optical axis, $n(x, y, z)$ is the 3D spatial distribution of the RI of the sample and n_m is the homogeneous RI of the surrounding medium. Indeed, QPI methods are a class of label free techniques that allow the 2D, 3D and 4D non-destructive imaging of completely transparent structures in a quantitative manner. Several QPI techniques are available [3]. In phase-referenced interferometry [4] and in spectral-domain phase microscopy [5, 6], the measured quantity is the phase delay at different points measured pointwise through scanning devices. Other QPI techniques allow to directly acquire the entire Field of View (FOV) of

the phase delay images, such as non-interferometric techniques like iterative I-O algorithms and Gerchberg-Saxton [7] and Transmission Intensity Eq. [8] based, Fourier ptychography and interferometric techniques as gradient light interference microscopy [9], spatial light interference microscopy [10] and DH. In this work the focus is on the latter, which represents the most affirmed QPI technique whose unique advantages will be highlighted in the following Paragraphs.

1.2 Principles of Digital Holographic Microscopy

Holography was invented by Gabor in 1948 [11] for finding an alternative solution to the problem of spherical aberrations in electron tomography. Holography allows to record the entire information contained in a complex wavefront, e.g., both the amplitude and the phase and, because of this powerful characteristic, it quickly become a general technique applied to many scientific fields [1]. A decisive step forward in this technology happened when it was converted from an analogic technique to a digital one, allowing to benefit from a robust numerical pipeline to record and process the holograms, initiating the era of DH. Holography is a general technique and can be applied to all the regions of electromagnetic spectrum, however, its main applications regard the use in the optical band for optical metrology purposes, in particular for microscopic imaging.

The holographic process consists of two steps: the hologram recording and its reconstruction. Concerning the hologram recording, since optical sensors only respond to light amplitude, the phase information is converted into amplitude's one through interferometry. The hologram is defined accordingly as the recording of the interference pattern between a reference wavefront, of known amplitude and phase, and an object wave [12]. The reference beam does not interact with the sample during its propagation. Generally, it is generated by a coherent light source such as a laser. The object wavefront instead is modulated by the interaction with the sample, as depicted in Fig. 1.1 (a). To mathematically describe the holographic recording process, three different approaches can be adopted [13], based on the Fresnel diffraction principle [14], the angular spectrum propagation [15] and the convolution [16, 17]. Here all the descriptions are reported, noting that the numerical implementation used in this thesis work is based

on the angular spectrum approach as it allows to obtain robust numerical results.

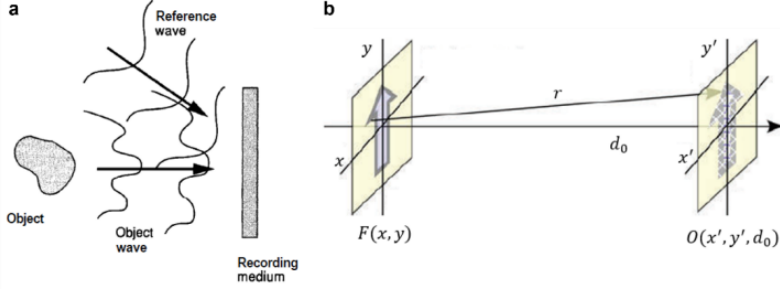


Figure 1.1. Hologram recording scheme. (a) Interference principle for holography between object and reference waves. (b) Geometric scheme for the holographic process description. Fig. (a) reproduced from reference [11], Fig. (b) reproduced from reference [12].

1.2.1 Discrete Fresnel diffraction transformation

Without loss of generality, we consider a coordinate system xyz centred on the object and represented in Fig. 1.1 (b). The xy plane is called object plane and acts as an aperture. The complex object wavefront emerging from the object plane can be generally written as

$$F(x, y) = F_0(x, y) \exp[j\phi_0(x, y)] \quad (1.2)$$

Where the phase $\phi_0(x, y)$ carries information about the sample. The object beam propagates along the z axis for the distance d_0 . At this z coordinate, called recording distance, the coordinates x' and y' individuate a plane called hologram plane, sketched in Fig. 1.1 (b). The diffracted field over the hologram plane can be expressed by the means of the Fresnel-Kirchhoff diffraction formula [13] as

$$O(x', y', d_0) = -\frac{j}{\lambda d_0} \int \int_{R^2} F(x, y) \exp\left[\frac{j2\pi}{\lambda} \sqrt{(x - x')^2 + (y - y')^2 + d_0^2}\right] dx dy \quad (1.3)$$

Considering the linearity of the expression, Eq. (1.3) can be expressed by the means of a convolution integral where the Point Spread Function (PSF) is the Huygens spherical wavelet S_H as described in (1.4) and (1.5)

$$O(x', y', d_0) = \text{conv}(F(x, y), S_H(x', y', d_0)) \quad (1.4)$$

$$S_H(x', y', d_0) = -\frac{j}{\lambda d_0} \exp\left[\frac{j2\pi}{\lambda} \sqrt{(x')^2 + (y')^2 + d_0^2}\right] \quad (1.5)$$

Assuming the paraxial approximation valid for

$$d_0^3 \gg \frac{\pi}{4\lambda} [(x - x')^2 + (y - y')^2]_{\max(x', y')}^2 \quad (1.6)$$

the PSF in Eq. (1.5) can be written as a Fresnel PSF as in (1.7)

$$S_F(x', y', d_0) = -\frac{j}{\lambda d_0} \exp\left[\frac{j2\pi d_0}{\lambda} + \frac{j\pi}{\lambda d_0} (x'^2 + y'^2)\right] \quad (1.7)$$

and therefore the diffraction integral at the hologram plane under the Fresnel approximation is expressed as

$$O(x', y', d_0) = \frac{-j}{\lambda d_0} \exp\left(j\frac{2\pi d_0}{\lambda}\right) \iint_{R^2} F(x, y) \exp\left[\frac{j\pi}{\lambda d_0} [(x - x')^2 + (y - y')^2]\right] dx dy \quad (1.8)$$

Finally, with some mathematical manipulation, the diffraction integral (1.8) is written as

$$\begin{cases} O(x', y', d_0) = Z(x', y', d_0) FT\{F(x, y)W(x, y, d_0)\} \\ Z(x', y', d_0) = \frac{j}{\lambda d_0} \exp\left[\frac{2\pi}{\lambda} \left[d_0 + \frac{x'^2 + y'^2}{2d_0}\right]\right] \\ W(x, y, d_0) = \exp\left\{j\frac{\pi}{\lambda d_0} (x^2 + y^2)\right\} \end{cases} \quad (1.9)$$

with operator FT representing the Fourier transform. The reference beam at the hologram plane is expressed as

$$R(x', y') = a_R \exp[j2\pi(u_R x' + v_R y')] \quad (1.10)$$

where u_R and v_R represent the spatial frequencies and a_R its amplitude. At the hologram plane the detector records the amplitude of the total wavefront incident over it. If the coherence conditions between the object and the reference wavefront are met, the interference between the two wavefronts allows to save the phase information in the interference fringes. Therefore, the amplitude of the sum between the reference and object beam at the hologram plane can be written as Eq. (1.11), where the $*$ symbol represents the complex conjugate

$$H(x', y', d_0) = |O(x', y', d_0)|^2 + |R(x', y')|^2 + R^*(x', y')O(x', y', d_0) + R(x', y')O^*(x', y', d_0) \quad (1.11)$$

which is the definition of the hologram H . In Eq. (1.11) three terms appear, called diffraction orders. The component

$$Q(x', y', d_0) = |O(x', y', d_0)|^2 + |R(x', y')|^2 \quad (1.12)$$

is called zero order term and accounts for the absolute values of the wavefronts only. Hence, it is what a normal photographic process would record if no interference was present. The two other terms expressed in eqs. (1.13) and (1.14) are called first order and conjugate order and carry a redundant information about the complex object wave, being one the complex conjugate of the other.

$$H^{+1}(x', y', d_0) = R^*(x', y')O(x', y', d_0) = a_R|O(x', y', d_0)|\exp[j\arg(O(x', y', d_0))]\exp(-j2\pi(u_Rx' + v_Ry')) \quad (1.13)$$

$$H^{-1}(x', y', d_0) = R(x', y')O^*(x', y', d_0) = (H^{+1}(x', y', d_0))^* \quad (1.14)$$

The aim of the numerical processing stage is to reconstruct the entire object wave starting from one of the two diffraction orders.

1.2.2 Angular Spectrum reconstruction method

Similarly, the entire hologram recording process can be described by the means of the angular spectrum approach [13]. The hologram reconstruction process implemented in this thesis follows this description which generally provides better numerical performance, as also commented in

[13]. Considering the same reference system as before and writing the object field as in Eq. (1.2), the angular spectrum at the object plane x, y is defined as the Fourier transform of the real object $F(x, y)$ according to Eq. (1.15), where k_x and k_y are the projections of the wave vector $\bar{k} = (k_x, k_y, k_z)$, $k_z = \sqrt{k^2 - k_x^2 - k_y^2}$ over the image plane.

$$A_0(k_x, k_y) = FT[F(x, y)] = \frac{1}{2\pi} \iint_{R^2} F(x, y) \exp[-j(k_x x + k_y y)] dx dy \quad (1.15)$$

Symmetrically, the object wavefront can be obtained inverting Eq. (1.15)

$$F(x, y) = FT^{-1}[A_0(k_x, k_y)] = \frac{1}{2\pi} \iint_{R^2} A_0(k_x, k_y) \exp\{j(k_x x + k_y y)\} dk_x dk_y \quad (1.16)$$

The propagation for a distance d_0 introduces a phase shift in the angular spectrum; accordingly, the object field can be written at the hologram plane as

$$\begin{aligned} O(x', y', d_0) &= \frac{1}{2\pi} \iint_{R^2} A_0(k_x, k_y) * \\ &* \exp(j(k_x x' + k_y y' + \sqrt{k^2 - k_x^2 - k_y^2} d_0)) \text{circ}\left(\frac{\sqrt{k_x^2 + k_y^2}}{k}\right) dk_x dk_y = \\ &= FT^{-1}(A_0(k_x, k_y) \exp(j(k_x x' + k_y y' + \sqrt{k^2 - k_x^2 - k_y^2} d_0)) \\ &\quad \text{circ}\left(\frac{\sqrt{k_x^2 + k_y^2}}{k}\right)) \end{aligned} \quad (1.17)$$

where the circle function *circ*, whose value is one where the argument is less than one and is zero otherwise, limits k_z to real only values. Again, Eq. (1.17) can be written as a convolution as in (1.18)

$$O(x', y', d_0) = \text{conv}(F(x, y), S_A(x', y', d_0)) \quad (1.18)$$

in which

$$S_A(x', y', d_0) = \frac{1}{2\pi} FT^{-1}[\exp[jkd_0 - \frac{j\lambda d_0}{4\pi}(k_x^2 + k_y^2)]] \quad (1.19)$$

Under paraxial approximation, the Fresnel transform and the Angular Spectrum methods are equivalent. If the paraxial approximation is not valid, the Angular Spectrum approach or the Fresnel Kirchhoff diffraction formula must be used.

1.2.3 Reconstruction by convolution approach

According to this description, the object field at distance d_0 , $O(x', y', d_0)$, can be written as [16, 17]

$$O(x', y', d_0) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} F(x, y) f(x, y, x', y') dx dy \quad (1.20)$$

where f can be approximated as [17]

$$f(x - x', y - y') = \frac{1}{j\lambda} \frac{e^{jk\sqrt{d_0^2 + (x-x')^2 + (y-y')^2}}}{\sqrt{d_0^2 + (x-x')^2 + (y-y')^2}} \quad (1.21)$$

Through Eq. (1.21) it can be seen that the linear system characterized by $f(x, x', y, y') = f(x - x', y - y')$ is space invariant, therefore, the integral Eq. (1.20) is a convolution. The convolution theorem can therefore be used, allowing to find the $O(x', y')$ as an inverse transform as in (1.22), in which FT represents the Fourier transform operator

$$O(x', y') = FT^{-1}\{FT\{F(x, y)\}FT\{f(x, y)\}\} \quad (1.22)$$

This method presents some advantages especially in the case in which the hologram plane does not coincide with the plane at which the image is acquired. In this case Eq. (1.20) is rewritten at the acquisition plane, say (x'', y'') with the advantage that the size of the reconstructed image does not change in respect to the hologram plane, $\Delta x' = \Delta x''$, $\Delta y' = \Delta y''$. Moreover, since an analytical expression for the Fourier transform of f is available, the convolutional method only requires to compute two Fourier

transforms to reconstruct the hologram.

1.3 Recording systems

A basic Digital Holographic Microscopy (DHM) setup consists of an illumination source, an interferometer, a digitising camera, and a computer [13]. As mentioned, a laser light is used as an illumination source with necessary coherence to produce interference, even though incoherent holography has been demonstrated and allows reconstructions of increased resolution [18]. Two different configurations of holography are possible, in-line and off-axis. In in-line holography, the original technique invented by Gabor, the reference and the object beams propagate along the same direction. This configuration is straightforward to implement, however, it results in a superimposition of the virtual image and the scattered light from the directly transmitted beam. The so-called twin image problem requires nontrivial numerical approaches to be solved and often result in a poor quality of the reconstructed image. In the off-axis configuration, depicted in Fig. 1.2, an appreciable angular tilt is introduced between the object and the reference waves [11]. This allows to separate the contributions in Fourier space of the three orders of diffraction [19].

Concerning the interferometers, two choices are most commonly em-

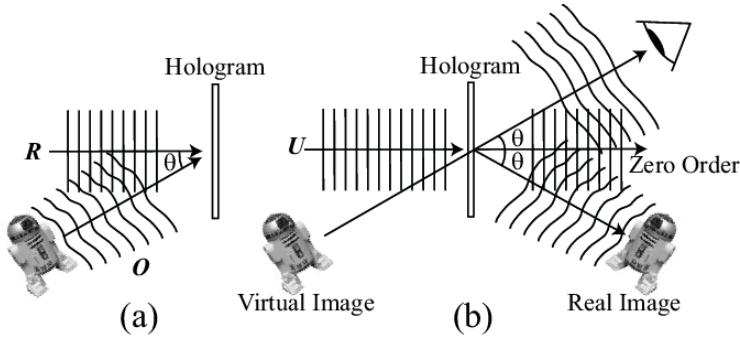


Figure 1.2. Off-axis holographic configuration. The twin image problem is illustrated, highlighting the advantages brought by the off-axis configuration in terms of angular separation of the contributions. Fig. reproduced from reference [20].

ployed. The first one is the Michelson interferometer, depicted in Fig. 1.3 (a), mainly used for reflective objects. The second one is the Mach-Zehnder, depicted in Fig. 1.3 (b), commonly used for transmission objects. Other choices are also possible, like the use of the Sagnac interferometer [21], illustrated in Fig. 1.3 (c). In all these designs the object is illuminated with a plane wave and the reference reaches the sensor plane with the same wavefront curvature as the object wave, except for an offset in the angle of incidence in off-axis configuration [13]. The Mach-Zehnder is the most commonly used interferometer even though it leads to some important drawbacks which will be discussed in Chapter 3. Concerning the recording device, a CCD or a CMOS camera are usually employed; the hologram is projected into the camera to be digitised and recorded. The pixel size of the cameras is several microns with pixel count up to tens of million, keeping in mind that the higher the pixel number, the larger the amount of data to store and the lower the maximum camera frame rate. These parameters, coupled to the properties of the Microscope Objective (MO), define the possible range of applications of the specific DHM setup.

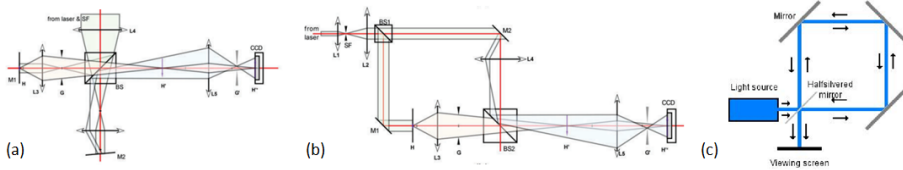


Figure 1.3. Principal interferometric configurations. (a) Michelson interferometer. (b) Mach-Zehnder interferometer. (c) Sagnac interferometer. Subfigures (a) and (b) reproduced from reference [22], subfigure (c) reproduced from reference [23].

1.4 Step-by-step holographic numerical reconstruction

As mentioned before, a numerical processing of the hologram is needed to extract the information related to the object beam by the Eq. (1.11). The reconstruction aims to extract the phase of the hologram as an image

termed **QPM**. The numerical procedure to obtain the **QPMs** has been standardised over time in the literature [13] and generally of several steps, nominally hologram preprocessing and apodization, hologram demodulating, refocusing, aberration compensation, unwrapping and occasionally a denoising stage. This pipeline is illustrated in Fig. 1.4 for the case of a monocyte from line THP1. This procedure can be however slightly changed and adapted to the specific setups in use.

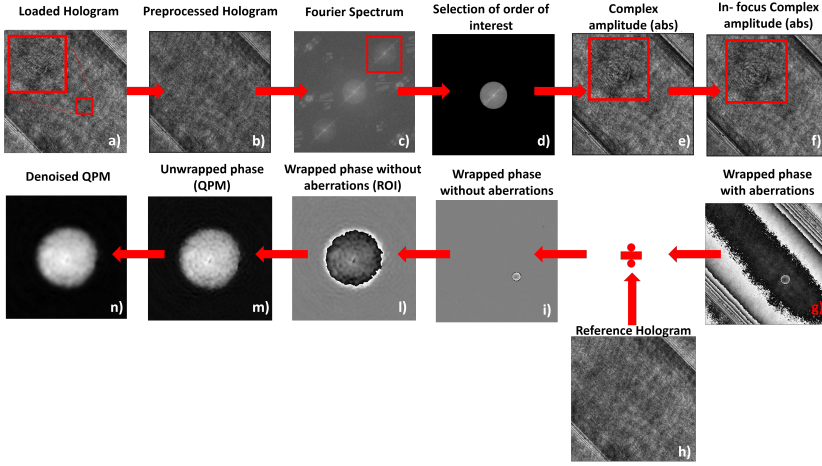


Figure 1.4. Sketch of the holographic processing pipeline. (a) Acquired hologram, the cell is highlighted in the red box. (b) Hologram after the preprocessing stage. (c) Fourier spectrum of the hologram. Thanks to the off-axis configuration, the three diffraction orders are separated. The diffraction order of interest is highlighted in the red box. (d) The selected order of diffraction is isolated and centered at the center of the hologram. (e) Amplitude of the demodulated hologram. (f) Refocused complex amplitude (abs). (g) Wrapped phase with aberrations. (h) Reference hologram used for the correction of the aberrations. (i) Phase of the sample aberrations-free. (l), Region of Interest (**ROI**) containing the wrapped phase. (m) Unwrapped phase (**QPM**). (n) Denoised **QPM**.

1.4.1 Apodization

The hologram preprocessing stage comprises a set of operations performed over the hologram to enhance its quality and allows a more comfort-

able tracking of the sample. The most important step of the preprocessing stage is apodization. This procedure is used to decrease the effects introduced by the sharp borders of the hologram aperture, which due to the of the diffraction effect introduce spurious fringing in the reconstructed holograms. This correction is numerically accomplished tapering the borders of the hologram with a smoothly attenuating function [24]. An example of preprocessed hologram is displayed in Fig. 1.4 (b).

1.4.2 Extraction of real order of diffraction

As mentioned above, Eq. (1.11) carries information about the three orders of diffraction. In off-axis configuration, three orders occupy distinct regions of the Fourier spectra, therefore the diffraction term of interest can be selected using a circular mask as a band-pass filter cantered around the order centre and with an opportune pupil. The choice of the radius of the circular mask is important as it determines the spectral contribution that will be preserved. The inverse Fourier transform of the filtered hologram is termed demodulated hologram or complex amplitude. The amplitude of the Fourier spectra of an hologram is showed Fig. 1.4 (c) while in subfigure (d) the filtered order of interest is depicted shifted at the center of the hologram. The demodulated hologram is displayed in Fig. 1.4 (e).

1.4.3 Automatic focusing

One of the most advantageous properties of DH is a direct consequence of the availability of the entire complex wavefront. Indeed, it is possible to acquire out of focus digital holograms and then numerically process them in an automated way to obtain a refocused version according to the mathematical descriptions provided in the previous chapters, with a process called autofocusing. To determine the focus distance of the imaged object different metrics have been proposed in the literature [25]; however, in this thesis the Tamura Coefficient (TC) [25] is used. This coefficient, whose expression is $TC(I) = \sqrt{\frac{\sigma(I)}{\mu(I)}}$, is evaluated over a ROI I extracted from the amplitude of the out-of-focus demodulated hologram centred around the imaged object's centroid [26]. In the TC expression, σ is the standard deviation of the amplitude of the ROI, whereas μ is its mean value. The evaluation of the TC over a discrete set of distances over the optical axis

z represents a metric that has shown great stability for the identification of the focusing plane, as it represents a convex functional whose global minima individuated the refocusing plane of the hologram. The focusing operation can be made separately for each object imaged in the FOV as long as the ROI selected for the evaluation of the TC contains just one of them. This can strongly increase the throughput of the imaging system, as long as the numerical refocusing procedure is efficient enough, since there is no need to physically establish an a-priori focusing distance and force the samples to place accordingly. An example of a numerically refocused hologram is displayed in Fig. 1.4 (f).

1.4.4 Aberration Compensation

The acquired numerical hologram contains aberrations introduced by the optical systems. Different approaches can be numerically implemented to compensate them [13, 27]. The most common techniques are fitting-based procedures [28] that estimate a mathematical model for the aberrations and then use it to remove their contribution from the hologram. Reference-based methods represent a valid alternative, according to which a reference hologram (e.g., an hologram not containing the specimen that only carries the aberrations contributions linked to the optical system) is used to remove them from the hologram. [29]. Generally, this approach is less computationally demanding than the mathematical fitting. Fig. 1.4 (g) shows an hologram corrupted with aberrations, while subfigure (h) shows an example of reference hologram. The effects of the aberration compensation step are displayed in Fig. 1.4 (i).

1.4.5 Phase Unwrapping

The aberration removal provides a wrapped phase image suffering of modulo 2π ambiguities, as every object whose optical thickness variations that exceed the wavelength introduces phase jumps. The 2D phase unwrapping operation is a non-trivial step and multiple approaches have been proposed in the literature. One of the most used unwrapping methods is the PUMA algorithm [30], based on the exact minimisation of an energy functional in the case of convex problems and on the approximate minimisation in the case of non-convex problems. Finally, the PUMA algorithm

is often coupled with error-correction routines able to fix the remaining errors that might still affect the phase map at the end of the unwrapping routine. The phase image containing the phase jumps is depicted in Fig. 1.4 (l), while the unwrapping result is shown in Fig. 1.4 (m).

1.4.6 Denoising

Finally, the last numerical stage used is a denoising stage. This is often performed to attenuate the speckle components of noise that are present over the phase map due to the coherent source of illumination. Denoising is a delicate stage as, being DH a quantitative imaging technique, it might comport a loss of information with metrological relevance carried by the QPMs. A performing denoising routine is based on a 2D windowed Fourier transform [31]. Fig. 1.4 (n) shows a denoised QPM. Note that, it is possible to perform the phase denoising also before applying the unwrapping algorithm.

1.5 Applications of DHM to biological imaging

DHM is a very effective process for achieving high-precision QPI in microscopy and comprehensive studies of its applications can be found in [32, 1]. DHM can be effectively used to study biophysical and pathophysiological properties of biological specimens. For example, QPI of Red Blood Cells (RBC) is widely studied as their phase delay can be directly transformed into volumetric information given their constant internal RI profile [33, 34, 35, 36, 37, 38, 39]. Information of their chemical properties can be obtained [40, 41, 42], together with the possibility to investigate their dynamic behaviour. In particular, the dynamic fluctuation of the morphology of their external membrane can be used to determine their pathophysiological states [43, 44, 45, 46, 47, 48, 49] or to study biomechanical properties [50, 51, 52]. Moreover, it was successfully used for the investigation of live neurons during their electrical activity [53, 54] [55] and to study the spontaneous contraction of cardiomyocytes [56]. Among the most impactful applications of QPI is measuring in a non-destructive fashion single cell mass and volume over arbitrary periods of time, both in adherent and in-flow conditions. In fact, QPI can be used to measure accurately the

growth rate of single cells [57, 58, 59]. QPI provides also the outstanding property to weight cells by simply imaging them, as their RI is proportionally related to cell density. In fact, from QPI it is possible to evaluate a significative parameter called Dry Mass (DM), which measures the density of the non-aqueous content of the cell, mainly determined by proteins and lipids [60]. DM $\sigma(x, y)$ can be obtained from the measured QPMs $\phi(x, y)$ as $\sigma(x, y) = \frac{\lambda}{2\pi\alpha}\phi(x, y)$, where α is called the refractive increment. The parameter α can be very reasonably estimated as 0.19 ml g^{-1} [61]. The DM of *E. coli* cells and human osteosarcoma *U2O2* cells has been measured for more than 60 minutes, and the cycle-dependency of *U2O2* cell growth has been reported [62]. Using the same method, the relationship between the motility and growth of S2 *Drosophila* cells under the influence of poly-L-lysine substrate has been investigated [63]. DM can also be used as an indicator for cell division [64]. Cell death, either as apoptosis or necrosis, can be determined by measuring the mean phase shift using QPI. This was proven for mouse cortical neurons, where the apoptosis was induced by applying L-glutamate, and the corresponding mean phase shift values have shown strong correlation with cell viability [65]. QPI has also been combined with laser microsurgery in order to evaluate the damage or repair of cells or organelles in real time during laser micro-dissection [66] and also been thoroughly used to study human diseases such as malaria or cancer. As many diseases can significantly alter the morphology of live cells, QPI offers important advantages over conventional imaging approaches [33, 3] in their characterisation. By measuring cell thickness using QPI, phenotyping of cancer cells was demonstrated [67]. Quantitative and label-free imaging capability makes QPI an effective method for blood screening, with promising diagnostics applications in the form of LB, which will be thoroughly discussed later on in this work. For example, the measured OPD information can be translated into haemoglobin (Hb) concentration for detecting sick RBC [68] and anaemias [69, 70]. Finally, exciting new possibilities of exploiting DHM arise from the possibility to fully make use of the 3D information QPMs convey, which is allowed by TPM. The basic principles and algorithms for tomography will be discussed in the next Chapters, with a focus on the applications in DH.

1.6 Digital Holographic techniques for biomedical imaging

Concerning the analysis and quantification of different aspects of cell biology, different high-throughput techniques are available. Among others, plate readers, sequencing platforms, DNA, RNA and protein microarrays, Western blotting are well affirmed ones used for this purpose. However, the drawback of these platforms is that they only allow to analyse at the cell population level without taking into account cellular intrapopulation heterogeneity in terms of cell cycle phase, size, volume, physiological state, and their individual development history. **FC** is a sophisticated technology that allows to measure at one time multiple physical characteristics of a single cell that flows in suspension through a measuring device. The measurements are linked to light scattering features of the samples under investigation, which may be derived from dyes or monoclonal antibodies targeting either extracellular molecules located on the surface or intracellular molecules inside the cell. Thanks to these features, **FC** represents a powerful tool for detailed analysis of complex populations in a short period of time thanks to the high-throughput property related to the flow condition [71].

The advent of **IFC** systems represented a remarkable enhancement of the **FC** potentialities. In **IFC** for each cell a bright-field image, a dark-field image, and several fluorescent images related to a specific marked intracellular component are acquired, extending the set of measurable single-cell parameters. The last frontier of **IFC** is the implementation of these systems in the label-free modality to overcome the drawbacks linked to the use of fluorescence tags, in particular incorporating **DH** imaging. Finally, the possibility to incorporate tomography into **DH**-based **IFC** systems, leading to the so-called **TPIFC** systems, allows to fully extract the information conveyed by **DH**. The first attempts to realise **TPM** of cells flowing in a microfluidic channel date back to 2014 [72], but the paradigm of **TPM** in flow modality was effectively demonstrated In 2017 at ISASI-CNR, realising for the first time **TPIFC** [73]. Summing up, **TPIFC** provide the 3D, label-free, and quantitative characterisation of single cells in high-throughput. In the following sections a brief introduction over **FC**, **IFC** and label-free **IFC** will be given, while the accurate description of the **TPM** and **TPIFC** techniques

will be object of discussion of the next chapters.

1.6.1 Flow Cytometry

FC is an advanced technology that allows to measure at one time multiple physical and biochemical characteristics of a single cell that flows in suspension through a measuring device. This is made measuring light scattering and fluorescence emission of the sample, which are collected through an optical excitation source which strikes the flowing particles as illustrated in Fig. 1.5. [74]. The major advantages offered by FC regard

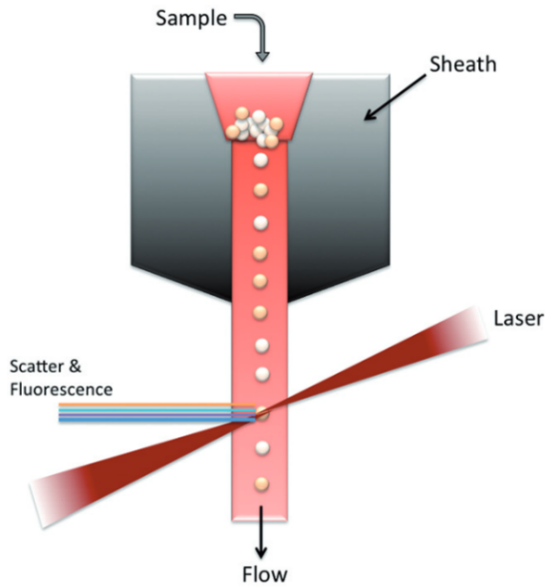


Figure 1.5. Sketch of a FC. Image reproduced from reference [71].

the possibility to acquire many information about a large, heterogeneous population while keeping the informativeness on a single-cell level. In addition, thanks to the in-flow modality, this technique offers an extremely high throughput. FCs were first introduced in late 1960 and could measure only one parameter, the size of the flowing cells. Since then, the technology has rapidly evolved and modern devices can measure up to 19 parameters at one time [75], with the possibility to also count and sort populations.

Nowadays, FC devices are essential tools frequently used for clinical and diagnostic purposes, allowing to investigate biological specimens up to the sub-cellular level, detecting internal structures such as organelles, protein, DNA and RNA as well as whole cellular processes such as cell proliferation, cell cycle and apoptosis [71]. Concerning the scattering measurements, the phenomenon is mainly related to the shape of the cell and its internal structure, so it gives access to its morphology. With a simplified description two major contributions of scattering can be differentiated [71]. The first one, which occurs in the same direction of the probing laser beam or with slight angular deflection, is termed forward scatter and is mainly related to the diffraction effect produced by the external shape of the cell. The second contribution, which occurs in a direction horizontally deflected from the laser beam direction (typically detected at 90 degrees), is termed side scatter and is mainly related to the reflection of the incident light caused by the internal structures of the cell. Concerning the fluorescence measurements, they allow to investigate the biochemical and internal structure of the cells according to the principle of light excitation. The samples are generally stained with fluorescent probes called fluorochromes that bind to the sample or one of its specific internal structure, thus revealing it. Fluorochromes are characterised by the spectrums of the absorbed and of the emitted light. If the wavelengths of the absorbed and emitted light are different, optical filters can be used to separate the two contributions. The use of different fluorescent agents at the same time allows the multiparametric analysis of FC, with different fluorochromes used to target different aspects. The main subsystems composing a FC device are the fluidic part, the optical part, and the electronics part. Concerning the fluidic part, it is responsible of directing the sample towards the probing apparatus in an ordered fashion in order to be scanned, as the samples are initially spread and randomly dispersed into the suspension media. This is made carefully controlling their position to make them align in a single file. This process, called hydrodynamic focusing, is regulated by a sophisticated pressurised control over the flowing samples and grants a uniform illumination for all of them. The injection rate of the cells into the microfluidic apparatus is controlled by the user of the device and depends on the measurement of interest. The optical subsystem is responsible of focusing the light on the sample to be probed and collecting the optical measurements performed

towards the electronic network, which is responsible for the analysis of the performed measurements. The illumination light is generally a laser source, whose characteristics determine the type of measurements that the device can perform. The collection optics allows to separate and categorise the contributions of scattered light and fluorescent emission. Regarding the scattered light, it is filtered according to the wavelengths composing it and then the various contribution are directed over appropriate detectors; the light contributions linked to fluorescence analysis are instead detected and differentiated using ad-hoc filters. Finally, the electronics subsystem is responsible for the detection of the optical contributions, which is performed through photodiodes or photomultiplier tubes, for the processing of the measured quantities to extract the morphological characteristic of interest and for their transmission to an external computational source. **FC** devices can be categorised in two main groups: the so-called non-sorters **FC**, which only measure properties of interest for the population under exam, and sorting **FC**, which can also physically separate the populations according to the measured properties. Among them, Fluorescence Activated Cell Sorting (**FACS**) are sorting **FC** devices which can sort fluorescent labelled cells among the entire population. Cell sorting is commonly made employing the so-called cell sorters units. These units operate using the electrostatic deflection principle of charged droplets. To obtain this, the flowing cells are organised into droplets which are then charged by a charging electrode. According to the sign of the charge which depends on the samples contained in the droplets, the droplets are deflected towards a specific direction. **FACS** instead can sort an heterogeneous mixture of fluorescent-tagged cells into multiple categories one cell at the time. This operation is performed by an operator which selects the fluorescent-tagged cells as they pass through a laser beam. The analysis that can be performed over **FC** data generally regard statistical distribution of the measured quantities, commonly described through histograms or scatter plots. These analyses can allow to statistically separate the populations setting thresholds over the measured quantities and then to obtain information about the parameters for a single group. Nowadays, **FC** are still object of massive research interest. In particular, one trend is to incorporate another analysis class together to the mentioned scatter and fluorescence ones, which is the use of the spectral analysis. This analysis could enrich

the pool of possible extracted information and overcome the limitations of multichromatic fluorescence detection, linked to the low specificity of the separated fluorescence colour channels due to reciprocal contamination of the channels [76]. Finally, another important trend for FC comes from the development of microscopic fluidic devices [77]. Microfluidics chips are devices in which small amounts of fluids ($10e - 9/10e - 18$) can be processed and manipulated in channels with very small dimensions; they can be effectively conjugated to FC devices obtaining the so-called microfluidic flow cytometers. These chip-based flow cytometers are cost and size-effective and portable when compared to conventional benchtop instruments which often are bulky and heavy. The use of these devices allows several advantages compared to standard FC devices. In particular, one of interest regards their ability to concentrate the solutions of fluids which generally are processed by an FC device. The latter, in fact, works with high volume of fluids that have a low concentration; whereas the first has the ability to reduce the volume of fluids scanned by concentrating the samples. This allows to significantly slim up the optical section of the FC device, which is delicate and expensive, as the need to focus the widely spread buffer solution is absent. This can be also particularly advantageous in applications as the search of extremely rare cells such as CTCs which appear as 1-10 for mL of fluid. In fact, high volumes of sparse fluids should be analysed by a conventional FC device, while the use of microfluidic FC can significantly speed up this process. The possibility to obtain microfluidic FC devices is a vivid research area which will constitute one of the future directions of this technology [77].

1.6.2 Image Flow Cytometry

As mentioned in the previous section, FC is a long-term established tool to perform cell analysis given the unique advantages it offers such as the extremely high throughput, with rates up to tenths of thousands of cells for second [78], conjugated with the multiparametric analysis at single cell scale. However, the information collected is of statistical relevance at a population level, and even though it indeed regards single-cell measurements, the identity of the specific sample is lost. Recently, the possibility to incorporate imaging techniques into the FC technology has been successfully demonstrated, leading to the so-called IFCs [79, 71, 80].

IFCs devices allow to record images of the single cells which flow into a FC environment, conjugating its high-throughput with a major single-cell specificity. With the enormous amount of information they allow to save, IFCs are remarkably revolutionising single cell analysis [71, 81]. In fact, analyses such as the search of CTCs could strongly benefit of the record of the morphology of the single cell, as a tumoral cell can often be differentiated according to her shape. Moreover, IFC devices allow to quickly build vast datasets, which allows to use ML and DL approaches to extract additional information with a quasi-real time processing [82]. IFC systems structure is similar to the one of FC, comprehending a fluidic, an optical and an electronic subsystem, with the addition of an imaging device, generally a CCD camera or a CMOS one [75, 80, 83, 84, 85, 86]. A massive research effort put into the development of imaging technologies allowing a constantly greater throughput [82, 87, 88, 89, 90, 91]. In fact, a trade-off between the acquisition speed and the image quality is often present, as conventional cameras need a finite time to record the images. Hence, the acquisition subsystem often constitutes a bottleneck in terms of throughput. Early instruments were able to acquire approximately 100 cells per second, whereas new devices can allow to record images at speeds of up to 10,000 cells per second [89], obtaining a throughput similar with the one of FC systems. Commercial IFC devices are already available on the market and being used in different contexts, such as the pioneering ImageStream and Flowsight developed by Millipore [92] and ImageStreamX Mk [82]; FlowCam, originally developed by Fluid Imaging Technologies (Yarmouth, ME, USA) to study oceanic plankton [93] or The Submersible Imaging FlowCytobot [94], with imaging systems generally providing a bright field, a dark field and multiple fluorescence images. Finally, the capability of FC systems to sort cells has been incorporated in IFC systems too, developing devices such as the intelligent Image-Activate cell sorters [95], where the AI is used over the acquired images to sort the samples. Nevertheless, the greatest bottleneck to the developing of IFC technology nowadays relies on the amount of information produced by a single realisation. Indeed, the biggest challenge lies in acquiring, storing, processing and quickly transferring a massive amount of images which can easily overcome tents of GB for a few minutes test, as the acquired data is much more complex than the one collected with conventional FC. The

solution of this problem is an active research field and progresses coming from the fields of compressive sensing and cloud computing [96] are being incorporated.

1.6.3 Label-free IFC

As discussed, the imaging of biological samples is commonly made employing fluorescent dyes or tags. Even though this technique is the state-of-the-art for microscopy [97, 98, 99], it has important drawbacks. The use of labels is often non compatible with live cell analysis as they alter their physiological behaviour. Photobleaching can compromise long-runs experiments and photodamaging could alter the cell life cycle. In addition, staining requires sophisticated chemical processes which are expensive and must be handled by specialised personnel. Nevertheless, the main drawback in the use of chemical labels is the need to know a-priori information about the sample object of imaging [100]. In fact, labels or stains often tackle a specific organelle or antigen which univocally identifies the sample of interest; if these identifiers are not known a-priori their tagging, and therefore the imaging, is not possible. This is a serious issue in all the applications where the detection of rare cells is needed, such as the search for CTCs in the bloodstream. Given their great inter variability, in fact, identifiers are often unknown or not enough descriptive. All these drawbacks make a label-free technique very attractive [101]. Among the existing label-free imaging methods, DHM is particularly interesting as it is a label free, quantitative technique. The acquired images, indeed, contain additional information which might enrich the pool of extracted features from the images. In addition, one of the most powerful advantages offered by DHM is the possibility to acquire images out of a pre-determined focus, as the record of the entire complex wavefront allow the numerical post-processing refocusing. This allow to slim up the instrumentation, to make the setup more robust and to increase the throughput of the acquisition as images of all the specimens in the FOV can be acquired at the same time. Accordingly, the conjugation between IFC and DH is an active research field. Currently, the major limitation for this technology is the trade-off between the camera's framerate and its sensitivity; therefore, today it is still difficult to obtain images of high quality with high throughput [102], and detailed information about the cell and its internal

structures are only obtained scaling the throughput of the system of a factor 100-1000. Nowadays, the state-of-the-art label-free IFC technique is a time-stretch interferometer microscope (namely multi-ATOM), which enables single-cell QPI at subcellular resolution operating at a relatively high cell imaging throughput (>10000 cells/s), at least 100 times faster than classical QPI but significantly slower than state-of-the-art FC devices [102, 100].

1.6.3.1 Towards lab-on-a-chip DHM-FC

Current commercial FC systems are still characterised by a high cost, large dimension, and the need of highly skilled personnel to handle and maintain them. A commercial FC device with sorting capabilities generally falls in the price range of 200k-1M dollars. Consequently, only well-founded, centralised labs have the possibility to acquire them. This is in contrast with the global tendency to go towards the paradigm of telemedicine [103], according to which healthcare will be always more distributed and easily accessible by common users. A central technology enabling this paradigm LOC devices, which will be cheap, robust, easy to handle, small systems equipped with a great diagnostic power [104, 105]. The research trend in clinical and healthcare is therefore to develop devices which can perform the described cellular analyses falling into this paradigm. In this scenario, the technology of LOC devices exploiting microfluidics and equipped with robust optical imaging systems such as lens-free DHM, is of great interest [106, 107]. Lens-free on chip DHM is an emerging technique that allows the imaging of biological samples within a large FOV without using bulky lenses or optical components. The resulting devices are therefore compact, cost effective and robust, meeting the need of the LOC paradigm. Commercial applications are already available [108] on the market with the device measuring around 15 cm^3 , weighting less than a kg and costing around 2.5k dollars. A strong research and industrial effort is being paid into the development and commercialisation of this technique [109].

Chapter 2

Tomographic Phase Imaging Flow Cytometry (TPIFC)

As mentioned in the previous Chapter, [QPMs](#) represent an integral measurements of the coupled contribution of [RI](#) profile and physical thickness of the sample. These quantities can be decoupled exploiting a tomographic-based technique termed [TPM](#), allowing to reconstruct the entire 3D [RI](#) profile of the cell under exam. For its possibility of providing an ample set of biophysical properties of the cells which would be unavailable otherwise, [TPM](#) quickly captured enormous attention and since the first realisation in 2006 it become a principal tool in optical metrology. Different configurations of [TPM](#) are feasible; however, one of the most promising is [TPIFC](#), demonstrated for the first time in 2017 at ISASI-CNR [73]. Indeed [TPIFC](#) allows to conjugate the fast throughput of [FC](#) devices with the single cell level specificity of [TPM](#).

This Chapter is structured as follows. First, the general description of tomography is given, both for straight-line approximation and for the diffraction description. Then, the principal reconstruction algorithms (Filtered Back-Projection ([FBP](#)) and Filtered Back-Propagation ([FBPR](#))) are described. Following, state-of-the-art solvers embedding regularisation are discussed, with a particular insight over [TV](#). Concluding, the [TPM](#) and [TPIFC](#) techniques are discussed in depth, presenting the experimental setup available at ISASI-CNR.

2.1 Introduction to Computed Tomography

Tomography is defined as the three-dimensional reconstruction of an object, modelled as a volumetric complex RI distribution $n(V)$, from integral images taken at different angles [110]. Although it is a general technique, one of the most common applications is Computed Tomography (CT), a tool to image 3D objects like portions of the human body. In CT $n(V)$ is reconstructed from transmission images at different orientations, i.e. from projections onto a two-dimensional (2D) detector plane, with the underlying assumption of straight line propagation. In fact, as the most organic tissues are characterised by an imaginary $n(V)$ profile, the major effect is the absorption of the incident light, without altering its propagation direction. Accordingly, the reconstruction of the object is an attenuation map $f(V)$ characterising it and the principal reconstruction algorithm used is the FBP. This algorithm, due to its simplicity, is also used in all the contexts in which, given the reduced resolution of the setups, the errors arising from neglecting the wave nature of the electromagnetic fields would not be appreciable. When the straight-line propagation is applicable, the tomographic process is termed *classical tomography*. In other cases, when the wavelength of the illumination beam is comparable to the size of the imaged object or the $n(V)$ profile has a non-neglectable real component, diffraction can not be overlooked anymore and the wave theory of light must be employed. In this case, instead of the projections, the quantity measured is the scattered field emerging from the sample at the detector, and the main reconstruction algorithm is called FBPR. This class of tomographic problems are termed *diffraction tomography*.

Considering the imaging of biological samples, tomography as introduced can be applied to them. As discussed, they are mainly characterised by a real $n(V)$ profile and do not absorb significant amounts of light; their effect is to induce a phase change into it. Consequently, imaging techniques that can quantify these sample-induced phase changes must be used, such as DHM. Indeed, the QPMs are integral measurements of a coupled contribution of the RI of the sample and its thickness, as expressed in Eq. (1.1); if QPMs relative to different orientations are available, exploiting suitable tomographic reconstruction algorithms it is possible to decouple the two contributions and reconstruct the RI profile. In the case in which

the effects of light scattering are discarded the imaging technique is termed **TPM**, whereas if they are embedded it is termed Optical Diffraction Tomography (**ODT**). The recorded set of measurements (projections, scattered field or **QPMs**), corresponding to different rotational positions of the object, constitute the so-called sinogram, which is the starting point of the reconstruction of the $n(V)$. Finally, the mathematical description of light propagation will follow a scalar theory, as coherent imaging and inverse scattering respond to this modelling [111].

2.1.1 Tomographic reconstruction algorithms

As mentioned, in the case of classical tomography a straight line propagation is supposed. This assumption leads to a dependence between the measured data at the detector, termed the projection, and the $n(V)$ ruled by the Radon transform [112]. This transform allows to identify a bijective correspondence between the Fourier transform of a projection and one spectral line of the Fourier transform of $n(V)$ according to the so-called Fourier Slice Theorem (**FST**). Based on this framework, the simple yet extremely used **FBP** algorithm can be derived. This reconstruction algorithm, however, might lead to strongly incorrect reconstructions when the diffraction phenomena cannot be neglected. If instead this effect is included in the modelling, supposing a first-order description using the Born or Rytov approximations, a correspondence can still be found between the Fourier transform of the measured scattered field and a frequential contribution of the $n(V)$ ruled by the Fourier Diffraction Theorem (**FDT**). In this way, an elegant correspondence between the **FST** and the **FDT** can be established. Starting from the **FDT**, in an analogous way to the case of **FBP** a simple reconstruction algorithm can be derived also in this case, called **FBPR**. Even though **FBPR** strongly improves the reconstruction in the cases where the **FBP** fails, its performance is still limited and therefore object of constant development [113]. Since then, different approaches for the inversion have been proposed, adding regularisation terms tackling hardware and physical limits [114, 115] to improve the quality of the reconstructions. The regularised approaches can be used as inversion techniques on their own or often paired to **FBP** and **FBPR** to overcome some of the limits they introduce. Among others, **TV** regularisation [116, 117] is one of the most used and provides optimal results when dealing with problems

such as limited number of measurements available [118] both in the cases of classical and diffraction tomography. Finally, with the advent of DL, data driven approaches have been included into the tomographic reconstruction, and an extensive overlook of these techniques will be provided in Chapter 4.

2.1.2 Straight ray approximation

As mentioned, under this approximation the relation between the measured data at the detector and the RI of the sample is established by the Radon transform, and the measured data are projections of the n -dimensional space of the sample into an $(n-1)$ dimensional integral space of the measurements. Here the derivations of the FST theorem and of the FBP algorithm are reported without loss of generalisation for a 2D case, noting that a 3D volume can be represented as a succession of 2D slices. However, the generalisation to a 3D description is straightforward. The geometry of the problem is illustrated in Eq. (2.1), in which a coordinate system (x, y) is centred over the object. The object is rotated

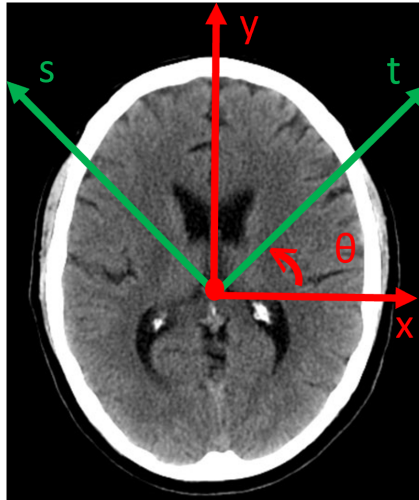


Figure 2.1. Reference system used for the FST and FBP algorithm derivation.

at each acquisition of an angle θ , and a coordinate system (s, t) solidal to this rotation is introduced. The two coordinate systems are related by Eq. (2.1):

$$\begin{cases} t = x\cos(\theta) + y\sin(\theta) \\ s = -x\sin(\theta) + y\cos(\theta) \end{cases} \quad (2.1)$$

Supposing a parallel beam illumination, the projection at the detector is computed as the line integral over a ray of the object of interest represented by the function $f(x, y)$ [112], as expressed by Eq. (2.2).

$$P_\theta(t) = \int_{(\theta, t)} f(x, y) ds \quad (2.2)$$

Which can be written in the (x, y) coordinate system as Eq. (2.3), where δ is the Dirac delta.

$$P_\theta(t) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} f(x, y) \delta(t - (x\cos(\theta) + y\sin(\theta))) dx dy \quad (2.3)$$

Defining $S_\theta(\omega)$ as the Fourier transform of the projection as in Eq. (2.4)

$$S_\theta(\omega) = \int_{-\infty}^{\infty} P_\theta(t) \exp(-j2\pi\omega t) dt \quad (2.4)$$

Eq. (2.4) can be rewritten as Eq. (2.5)

$$\begin{aligned} S_\theta(\omega) &= \\ &= \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} f(x, y) \delta(t - x\cos(\theta) + y\sin(\theta)) \exp(-j2\pi\omega t) dx dy dt = \\ &= \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} f(x, y) \int_{-\infty}^{\infty} \delta(t - x\cos(\theta) + y\sin(\theta)) \exp(-j2\pi\omega t) dx dy dt = \\ &= \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} f(x, y) \exp(-j2\pi\omega(x\cos(\theta) + y\sin(\theta))) dx dy = \\ &= F(\omega\cos(\theta), \omega\sin(\theta)) \end{aligned} \quad (2.5)$$

Having indicated with $F(\omega \cos(\theta), \omega \sin(\theta))$ the Fourier transform of $f(x, y)$. Eq. (2.5) is the statement of the fundamental theorem in tomography **FST**, which dictates that the Fourier transform of a projection at angle θ is distributed along a line at the same angle in the Fourier space of the object function. This theorem constitutes the basic theorem of the tomographic inversion, as the object of interest can be recovered as an inverse Fourier transform of the transformed projection. Generally, the inversion of Eq. (2.5) is not performed directly applying the inverse Fourier transform, as the filling in the Fourier space is made in a polar grid and the use of the inverse Fourier transform algorithms would generally require an interpolation over a rectangular grid, slowing down the inversion and introducing noise. Differently, another fundamental algorithm is chosen, **FBP**, which is easily derived mathematically manipulating Eqs. (2.5).

2.1.2.1 Filtered Back-projection

To derive the **FBP** algorithm, the starting point is to express the $f(x, y)$ as the inverse 2D Fourier transform of its Fourier transform $F(u, v)$.

$$f(x, y) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} F(u, v) \exp(j2\pi\omega(ux + vy)) du dv \quad (2.6)$$

Which can be written in polar coordinates as

$$f(x, y) = \int_0^{2\pi} \int_0^{\infty} F[\omega \cos(\theta), \omega \sin(\theta)] \exp(j2\pi\omega[x \cos(\theta) + y \sin(\theta)]) d\omega d\theta \quad (2.7)$$

with simple mathematical manipulations it is possible to get to the formulation in Eq. (2.8)

$$f(x, y) = \int_0^{2\pi} \int_0^{\infty} |\omega| F(\omega, \theta) \exp(j2\pi\omega(x \cos(\theta) + y \sin(\theta))) d\omega d\theta \quad (2.8)$$

Which is the formulation of the **FBP** algorithm and allows us to compute the object function with a series of sequential steps:

1. The projection at a given angle θ is acquired.
2. The Fourier transform of the projection $S_\omega(\theta)$ is computed, which for the [FST](#) is equal to $F(\omega, \theta)$.
3. $S_\omega(\theta)$ is filtered through $|\omega|$.
4. The filtered projection is Fourier inverted.
5. The contribution of all the projections is summed to obtain the reconstruction of $f(x, y)$.

This algorithm, yet very simple and easy to implement, is the base of the majority of the Radon inversion techniques due to its very low computational burden, providing a time and resource saving tomographic algorithm. This, for example, is often required in the case of sample rotation [TPM](#) for portable and real time applications, in which given the completeness of the input data with no missing cone effects it is possible to employ less powerful reconstruction algorithms with a consistent gain in terms of reconstruction speed and resource use.

2.1.3 Wave approximation

When the wavelength of the imaging beam is comparable to the size of the imaged object, diffraction cannot be neglected anymore, and a more sophisticated mathematical modelling of the process must be provided [\[110\]](#). The acquisition problem is described in [Fig. 2.2](#). A plane wave $u_0(\mathbf{r})$ with a wavelength λ in the visible spectrum illuminates the sample characterised by a [RI](#) distribution $n(\mathbf{r})$. The emerging field at a detector $u(r)$ is recorded for each rotation angle θ , with its phase data containing the information about $n(\mathbf{r})$. The scattering problem is generally described by the Helmholtz equation reported in [Eq. \(2.9\)](#)

$$(\nabla^2 + k(\mathbf{r})^2)u(\mathbf{r}) = 0 \tag{2.9}$$

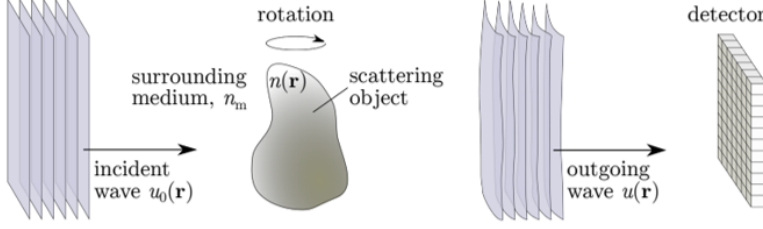


Figure 2.2. Geometry considered for the modelling of the tomographic problem when scattering effects are included. Image reproduced from reference [110].

in which $k(\mathbf{r})$ follows the expression

$$\begin{aligned}
 k_m &= \frac{2\pi n_m}{\lambda} \\
 n(\mathbf{r}) &= n_m + \epsilon_n(\mathbf{r}) \\
 k(\mathbf{r}) &= k_m \frac{n(\mathbf{r})}{n_m} = k_m \left(1 + \frac{\epsilon_n(\mathbf{r})}{n_m}\right)
 \end{aligned} \tag{2.10}$$

where n_m is the RI of the surrounding media and $\epsilon_n(\mathbf{r})$ is the RI variation respect n_m . In the inhomogeneous case, Eq. (2.9) can be expressed as

$$\begin{aligned}
 (\nabla^2 + k_m^2)u(\mathbf{r}) &= -f(\mathbf{r})u(\mathbf{r}) \\
 f(\mathbf{r}) &= k_m^2 \left[\left(\frac{n(\mathbf{r})}{n_m} \right)^2 - 1 \right]
 \end{aligned} \tag{2.11}$$

in which $f(\mathbf{r})$ is called scattering potential and k_m is the propagation constant in the homogeneous surrounding media; in the absence of the scatterer, so for $\epsilon_n(\mathbf{r}) = 0$, Eq. (2.9) is instead expressed in its homogeneous form which rules the propagation of the incident field $u_0(\mathbf{r}) = a_0 \exp(jk_m \mathbf{s}_0 \cdot \mathbf{r})$ as in Eq. (2.12)

$$(\nabla^2 + k_m^2)u_0(\mathbf{r}) = 0 \tag{2.12}$$

In the expression of the incident field, s_0 is the propagation vector for u_0 , a_0 is its amplitude and $\mathbf{r}$ is the propagation direction. Here, the convention

used is to gain a positive phase shift as the wave propagates in the positive direction of the chosen reference system. The solution to Eq. (2.11) can be found resorting to the Green function $G(\mathbf{r})$ in 3D defined as

$$\begin{aligned} (\nabla^2 + k_m^2)G(\mathbf{r} - \mathbf{r}') &= -\delta(\mathbf{r} - \mathbf{r}') \\ G(\mathbf{r} - \mathbf{r}') &= \frac{\exp(jk_m|\mathbf{r} - \mathbf{r}'|)}{4\pi|\mathbf{r} - \mathbf{r}'|} \end{aligned} \quad (2.13)$$

Resorting to the sampling property of the Dirac delta an integral equation for the field can be derived

$$u(\mathbf{r}) = \int G(\mathbf{r} - \mathbf{r}')f(\mathbf{r}')u(\mathbf{r}')d\mathbf{r}'^3 \quad (2.14)$$

Eq. (2.14) is an implicit equation in $u(\mathbf{r})$. The solution is nontrivial as no analytical forms exist. The way to approach this equation is to introduce some approximations to solve it.

2.1.3.1 Born approximation

Born approximation is a simple approximation which conveys an intuitive physical explanation. Subtracting Eqs. (2.11) and Eq. (2.12) an equation for the scattered component of the field $u_s(\mathbf{r}) = u(\mathbf{r}) - u_0(\mathbf{r})$ is obtained:

$$(\nabla^2 + k_m^2)u_s(\mathbf{r}) = -f(\mathbf{r})u(\mathbf{r}) \quad (2.15)$$

Which can again be solved by the means of the Green function in Eq. (2.13) up to obtaining an iterative expression solving for $u_s(\mathbf{r})$

$$u_s(\mathbf{r}) = \int G(\mathbf{r} - \mathbf{r}')f(\mathbf{r}')u(\mathbf{r}')d\mathbf{r}'^3 \quad (2.16)$$

The Lippmann-Schwinger equation for the total field $u(\mathbf{r})$ is written as [119]:

$$u_s(\mathbf{r}) = u_0(\mathbf{r}) + \int G(\mathbf{r} - \mathbf{r}')f(\mathbf{r}')u(\mathbf{r}')d\mathbf{r}'^3 \quad (2.17)$$

To force the Born approximation, the total field $u(\mathbf{r})$ is written as a series of functions called the Born series where the electric field is expanded in a series of terms proportional to successively higher powers of the refractive

index [119, 120] as in Eq. (2.18).

$$u(\mathbf{r}) = u_0(\mathbf{r}) + u_1(\mathbf{r}) + u_2(\mathbf{r}) + u_3(\mathbf{r}) + \dots \quad (2.18)$$

The first order Born approximation consist of using only the first term for this expansion, so, to approximate the total field with the incident field into the integral neglecting the scattered component.

$$u(\mathbf{r}) \approx u_0(\mathbf{r}) \quad (2.19)$$

Therefore

$$\begin{aligned} \int G(\mathbf{r} - \mathbf{r}') f(\mathbf{r}') [u_0(\mathbf{r}') + u_s(\mathbf{r}')] d\mathbf{r}'^3 &\approx \\ \int G(\mathbf{r} - \mathbf{r}') f(\mathbf{r}') [u_0(\mathbf{r}')] d\mathbf{r}'^3 &\end{aligned} \quad (2.20)$$

The solution to the Eq. (2.17) under the Born approximation is written as

$$\begin{aligned} u(\mathbf{r}) &\approx u_0(\mathbf{r}) + u_B(\mathbf{r}) \\ u_B(\mathbf{r}) &= \int G(\mathbf{r} - \mathbf{r}') f(\mathbf{r}') [u_0(\mathbf{r}')] d\mathbf{r}'^3 \end{aligned} \quad (2.21)$$

The Born series allows to give clear physical description of the scattering process. The first term of the series, corresponding to the incident field, represents the field strength that would be measured at the detector if the medium contained no irregularities. The second term of the series represents the single scattering phenomena, so the scattering of an incident plane wave over an irregularity in the point $\mathbf{r}$ with the scattered component reaching the detector as a spherical wave. The third term of the series represents the second order scattering phenomena and so on, as sketched in Fig. 2.3. Concerning the validity of the Born approximation, it clearly holds for the case in which the contributions of the scattered components are small when compared to the incident field,

$$u_s(\mathbf{r}) \ll u_0(\mathbf{r}) \quad (2.22)$$

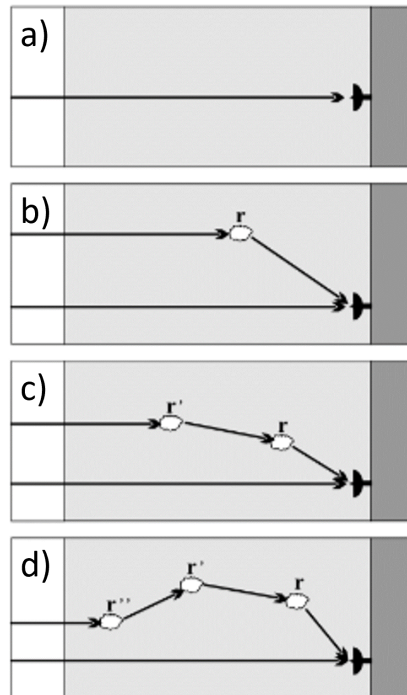


Figure 2.3. Higher orders modellings of the scattering phenomena. (a) Absence of scattering. (b) First order scattering approximation (Born). (c) Second order scattering approximation. (d) Third order scattering approximation. Image reproduced from reference [120].

Being these two quantities complex, the approximation is valid in the case of (i) little absorption by the specimen and (ii) small phase change into the sample. Since the absorption for biological specimens is always small and the imaginary part of the RI is irrelevant, the condition (i) is always verified. This is not true for the (ii). Following the calculations reported by [110] the validity of the Born approximation is verified when the overall phase change is smaller than 2π . This translates into a restriction for the optical path difference $s(n_s - n_m)$ which must be smaller than the wavelength λ . This is often non valid for thick biological samples such as whole cells. Therefore, despite its intuitive advantages, the Born approximation suffers from a serious limitation when applied to biological problems of scattering and more sophisticated approximations have to be used.

2.1.3.2 Rytov approximation

The Rytov approximation is a more powerful approximation compared to the Born one and can deeply extend the validity range of the latter. It is obtained starting from a manipulation of the inhomogeneous wave equation in Eq. (2.11) obtained supposing an exponential structure of the fields. In particular, the total field $u(\mathbf{r})$, which accounts for the incident and the scattered component, is written in an exponential form

$$u(\mathbf{r}) = \exp[\phi(\mathbf{r})] = u_0(\mathbf{r}) + u_s(\mathbf{r}) \quad (2.23)$$

where

$$\phi(\mathbf{r}) = \phi_0(\mathbf{r}) + \phi_s(\mathbf{r}) \quad (2.24)$$

writing the incident field as

$$u_0(\mathbf{r}) = \exp[\phi_0(\mathbf{r})] \quad (2.25)$$

then the total field is expressed as

$$u(\mathbf{r}) = \exp[\phi_0(\mathbf{r})]\exp[\phi_s(\mathbf{r})] \quad (2.26)$$

the introduced phases are complex quantities with a real component accounting for the attenuation in the propagation and the phase accounting

for delay.

$$\begin{aligned}\phi(\mathbf{r}) &= j\psi(\mathbf{r}) + \ln(a(\mathbf{r})) \\ \phi_0(\mathbf{r}) &= j\psi(\mathbf{r}) + \ln(a_0(\mathbf{r}))\end{aligned}\tag{2.27}$$

Accordingly, $u_s(\mathbf{r})$ is written as

$$u_s(\mathbf{r}) = u(\mathbf{r}) - u_0(\mathbf{r}) = \exp[\phi_0(\mathbf{r})][\exp(\phi_s(\mathbf{r}) - 1)]\tag{2.28}$$

Forcing Eq. (2.28) into Eq. (2.11) we get

$$(\nabla^2 + k_m^2)\exp[\phi(\mathbf{r})] = -f(\mathbf{r})\exp[\phi(\mathbf{r})]\tag{2.29}$$

Solving the computations in Eq. (2.29) we get a differential equation for the phase

$$\begin{aligned}\exp[\phi(\mathbf{r})][\nabla^2\phi(\mathbf{r}) + (\nabla\phi(\mathbf{r}))^2 + k_m^2] &= -f(\mathbf{r})\exp[\phi(\mathbf{r})] \\ [\nabla^2\phi(\mathbf{r}) + (\nabla\phi(\mathbf{r}))^2 + k_m^2] &= -f(\mathbf{r})\end{aligned}\tag{2.30}$$

A similar expression can be obtained for the phase of the incident field starting from Eq. (2.12)

$$\nabla^2\phi_0(\mathbf{r}) + (\nabla\phi_0(\mathbf{r}))^2 + k_m^2 = 0\tag{2.31}$$

Forcing Eq. (2.24) into Eq. (2.30) and exploiting Eq. (2.31) we get

$$\nabla^2\phi_s(\mathbf{r}) + 2\nabla\phi_s(\mathbf{r})\nabla(\phi_0(\mathbf{r})) + (\nabla\phi_s(\mathbf{r}))^2 = -f(\mathbf{r})\tag{2.32}$$

Multiplying this equation for the incident field $u_0(\mathbf{r})$ we finally get

$$(\nabla^2 + k_m^2)u_0(\mathbf{r})\phi_s(\mathbf{r}) = -u_0(\mathbf{r})[(\nabla\phi_s(\mathbf{r}))^2 + f(\mathbf{r})]\tag{2.33}$$

The Rytov approximation consists in forcing the term in square brackets in Eq. (2.33) to be equal to the scattering potential only, which is demanding that the gradient of the scattered phase is small respect to the perturbation. Accordingly, the equation is rewritten for the scattered phase under the Rytov approximation $\phi_s(\mathbf{r}) = \phi_R(\mathbf{r})$ as

$$(\nabla^2 + k_m^2)u_0(\mathbf{r})\phi_R(\mathbf{r}) = -u_0(\mathbf{r})f(\mathbf{r})\tag{2.34}$$

Eq. (2.34) can be solved again resorting to the Green function in Eq. (2.13) as the mathematical structure is corresponding to Eq. (2.15), therefore we obtain an equation for the complex Rytov phase $\phi_R(\mathbf{r})$

$$\begin{aligned} u_0(\mathbf{r})\phi_R(\mathbf{r}) &= \int G(\mathbf{r} - \mathbf{r}')f(\mathbf{r}')u_0(\mathbf{r}')d\mathbf{r}'^3 \\ \phi_R(\mathbf{r}) &= \frac{\int G(\mathbf{r} - \mathbf{r}')f(\mathbf{r}')u_0(\mathbf{r}')d\mathbf{r}'^3}{u_0(\mathbf{r})} \end{aligned} \quad (2.35)$$

Observing this equation, it is possible to see that the Rytov phase can be computed starting from the Born approximation of the scattered field and vice versa with just a manipulation of the data. This has interesting consequences from the reconstruction point of view, as the Rytov approximated data can be used in a Born-inversion framework with just a mathematical manipulation as ruled by Eqs. (2.36)

$$\begin{aligned} \phi_r(\mathbf{r}) &= \frac{u_B(\mathbf{r})}{u_0(\mathbf{r})} \\ u_R(\mathbf{r}) &= u_0(\mathbf{r})[\exp(\frac{u_B(\mathbf{r})}{u_0(\mathbf{r})}) - 1] \\ u_B(\mathbf{r}) &= u_0(\mathbf{r})\ln(\frac{u_R(\mathbf{r})}{u_0(\mathbf{r})} + 1) = u_0(\mathbf{r})\phi_R(\mathbf{r}) \\ u(\mathbf{r}) &\approx^{Born} u_0(\mathbf{r}) + u_B(\mathbf{r}) \\ u(\mathbf{r}) &\approx^{Rytov} u_0(\mathbf{r}) + u_R(\mathbf{r}) \end{aligned} \quad (2.36)$$

Concerning the validity of the Rytov approximation, following the calculations reported in [110] we can derive a mathematical expression

$$\begin{aligned} \frac{|\nabla\phi_s(\mathbf{r})|}{2\pi} &<< \frac{\sqrt{2n_m|\epsilon_n(\mathbf{r})|}}{\lambda} \\ \frac{|d\phi_s(\mathbf{r})|}{2\pi} &<< \frac{\sqrt{2n_m|\epsilon_n(\mathbf{r})|}|d\mathbf{r}|}{\lambda} \end{aligned} \quad (2.37)$$

Concluding, we require that the sample induces a phase change over a period of 2π radians that must be smaller than the variation in $n(\mathbf{r})$ along the corresponding optical path scaled by λ . The validity of the Rytov approximation is not dependent on the absolute phase change introduced

by the sample as in the Born approximation, but on the gradient of the RI within the sample. This makes the Rytov approximation more suitable for imaging of cells or thicker samples with a more complex inner structure.

2.1.3.3 Fourier Diffraction Theorem

Through the presented Born and Rytov approximations it is possible to link the measured scattered field at the detector and the $n(\mathbf{r})$ distribution of the sample. To proceed with the reconstruction, an equivalent to the Eq. (2.5) must be introduced. This is here described in a 2D case according to the geometry of Fig. 2.4.

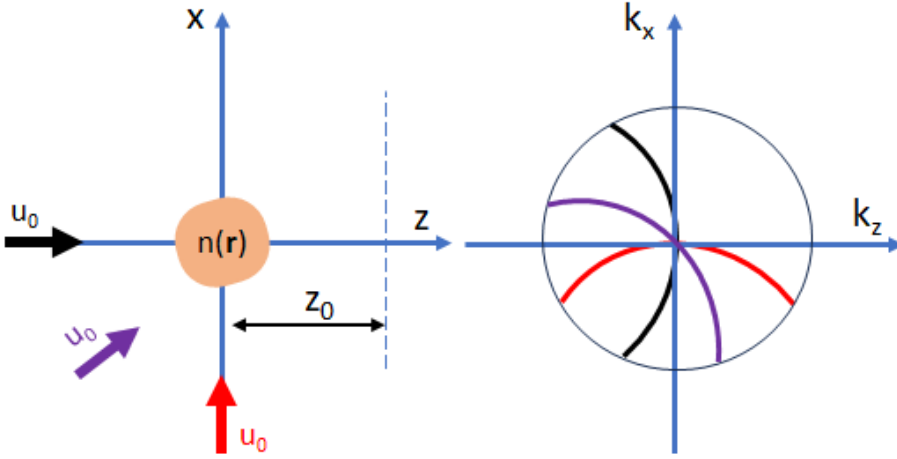


Figure 2.4. Geometry used for the derivation of the FBPR algorithm.

We define the 2D Green function as

$$G(\mathbf{r}, \mathbf{r}') = \frac{j}{4\pi} H_0^{(1)}(k_0 |\mathbf{r} - \mathbf{r}'|) \quad (2.38)$$

which can be expanded through the Weyl formula as

$$G(\mathbf{r}, \mathbf{r}') = \frac{j}{4\pi} \int ds_x \frac{1}{s_z} \exp[jk[s_x(x - x') + s_z(z - z')]] \quad (2.39)$$

In which we write the transverse wavevector $k_x = ks_x$ and the longitudinal

one $k_z = ks_z$ with (s_x, s_z) being the unitary vector. If we plug Eq. (2.39) into Eq. (2.21) we obtain

$$\begin{aligned} u_s(\mathbf{r}) &= \frac{1}{4\pi s_z} \int d\mathbf{r}' F(\mathbf{r}') u_0(\mathbf{r}') \exp[jk[s_x(x - x') + s_z(z - z')]] = \\ &= \frac{\exp[jks_z z]}{4s_z} \int ds_x \int d\mathbf{r}' F(\mathbf{r}') u_0(\mathbf{r}') \exp[-j\mathbf{k}\mathbf{s} \cdot \mathbf{r}'] \exp[jks_x x] \end{aligned} \quad (2.40)$$

If we write the scattered field as the inverse Fourier transform of its frequential expression $\tilde{u}_s(ks_x, z)$ with respect the coordinate s_x as

$$u_s(\mathbf{r}) = \frac{k}{2\pi} \int ds_x \tilde{u}_s(ks_x, z) \exp[jks_x x] \quad (2.41)$$

we can recognise Eq. (2.41) into Eq. (2.40) and therefore obtain Eq. (2.42)

$$\tilde{u}_s(ks_x, z) = \frac{j\exp[jks_z z]}{2ks_z} \int d\mathbf{r}' F(\mathbf{r}') u_0(\mathbf{r}') \exp[-j\mathbf{k}\mathbf{s} \cdot \mathbf{r}'] \quad (2.42)$$

writing $u_0 = \exp[j\mathbf{k}\mathbf{s}_0 \cdot \mathbf{r}] = \exp[jk(s_{0x}x + s_{0z}z)]$, where s_{0x} and s_{0z} are the counterparts of s_x, s_z for the incident field and measuring at the detector position $z = z_0$, Eq. (2.42) becomes

$$\tilde{u}_s(ks_x, z_0) = \frac{j\exp[jks_z z_0]}{2ks_z} \int d\mathbf{r}' F(\mathbf{r}') u_0(\mathbf{r}') \exp[-j\mathbf{k}(\mathbf{s} - \mathbf{s}_0) \cdot \mathbf{r}'] \quad (2.43)$$

The right-hand-side of Eq. (2.43) is nothing but the shifted Fourier transform of the scattering potential, which finally leads to

$$\tilde{F}[k(\mathbf{s} - \mathbf{s}_0)] = -2j\exp[jks_z z_0] ks_z \tilde{u}_s(ks_x, z_0) \quad (2.44)$$

Which is the statement of the [FDT](#) or the Wolf transform. This theorem states that when the object is illuminated from a direction $\mathbf{s}_0$ and the scattered field measured at all the possible directions $\mathbf{s}$, the latter gives information on the Fourier components $\tilde{F}$ whose points lie on a sphere of radius k centred in $-\mathbf{k}s_0$, called Ewald sphere for a 3D problem, or a circle, in the case of the 2D presented version, evidenced in Fig. 2.4. Therefore, it establishes a correspondence with the [FDT](#) and can be used to compute the scattering potential inverting this relation. In addition, a mathemat-

ical approach similar to the one used for the derivation of the FBP can be established leading to an algorithmic inversion of the Wolf transform called FBPR. Since FBPR, conversely to FBP, is out of the scope of this work, the derivation is not reported but can be found in [110]. Clearly, full coverage measurements are unfeasible to obtain, in addition, for technological reasons the angular range scannable is often limited. This brings to an incomplete frequencial filling generating a problem called missing cone. The correspondence between the FST and the FDT is explained in Fig. 2.5.

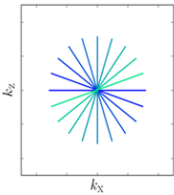
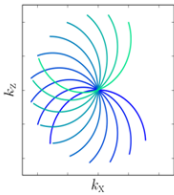
	Fourier Slice Theorem	Fourier Diffraction Theorem
	$\widehat{F}_{\varphi 0}(k_x, k_z) = A \sqrt{\frac{1}{2\pi}} \widehat{P}_{\varphi 0}(k_{Dx})$	
Fourier space coverage (180°)		
Sinogram $\widehat{P}_{\varphi 0}(k_{Dx})$	Fourier transform of projections $\widehat{P}_{\varphi 0}(k_{Dx})$	Fourier transform of complex scattered waves $\widehat{U}_{B,\varphi 0}(k_{Dx})$
Factor A	$A = 1$	$A = -\frac{2j k_m M}{a_0} \exp(-jk_m M l_D)$
Coordinates (k_x, k_z) sliced at φ_0	$\begin{cases} k_x = k_{Dx} \\ k_z = k_t = 0 \end{cases}$ Straight line	$\begin{cases} k_x = k_{Dx} \\ k_z = \sqrt{k_m^2 - k_{Dx}^2} - k_m \end{cases}$ Semicircular arc

Figure 2.5. Correspondence between the FST and FDT. Image reproduced from reference [110].

2.1.4 Regularized inversion

Both the [FST](#) and the [FDT](#) theorems in their [FBP](#) and [FBPR](#) forms are the fundamentals of the tomographic reconstruction but are often non employable for the poor performance they provide when inverting with noisy or undersampled data, filling the reconstruction with severe artefacts. Working with undersampled data is however a frequent condition, often sought after to reduce the acquisition time. At this purpose, more powerful numerical inversion techniques have been successfully introduced in the field of tomography [[121](#), [118](#), [122](#), [116](#), [117](#), [123](#), [60](#)], in which regularisation constraints are added in the reconstruction to increase its quality. Generally, the tomographic inversion problems are ill-posed and strongly benefit of the theory of regularisation to try to limit the effects of the ill-posing. The tomographic problem is rewritten as the functional $J(f)$

$$J(f) = \|Af - g\|_2^2 + \mu R(f) \quad (2.45)$$

where A is called the tomographic matrix and represents the forward operator accounting for the physical and geometrical modelling of the problem, f is the function we wish to reconstruct, g is the acquired measurement, $R(f)$ is a regularisation constraint whose function is to incorporate the a-priori information known about f to reduce the space of the searched solution and to counteract for problems induced by the inversion, and μ is called regularisation constraints and is used to establish a balance between the relative strength of the data fidelity and regularisation terms. The objective of the reconstruction is to minimise the functional $J(f)$. Many regularisation terms can be used, and a comprehensive discussion is made in [[114](#)]; however, one that plays a prominent role in the field of tomography is the [TV](#) minimisation.

2.1.4.1 TV minimisation

[TV](#) minimisation has gained a huge popularity since it was first introduced by Rudin, Osher and Fatemi in [[124](#)] as a denoising technique [[124](#), [125](#)]. Later, its benefits were recognised in different contexts such as deconvolution [[126](#), [127](#)], deblurring, image restoration [[128](#), [129](#), [130](#), [131](#)] and more generally as an inversion technique, making [TV](#) one of the most used solvers in the literature [[132](#), [130](#), [133](#)]. Since then, [TV](#) has been suc-

cessfully applied over different formulations of the tomographic problem, mainly in the field of [ODT](#), to help mitigate the problems of elongations over the optical axis [\[116\]](#). According to the [TV](#) formulation, the chosen regularisation term is the l_1 norm of the gradient of the searched solution, called the [TV](#) norm. Hence, including the [TV](#) regularisation, Eq. (2.45) is modified as in Eq. (2.46)

$$J(f) = \|Af - g\|_2^2 + \frac{\mu}{2} \|\nabla f\|_1 \quad (2.46)$$

The advantages of the use of [TV](#) regularisation are multiple. The l_1 norm is a sparsifying term, promoting the sparsity of the searched solution in an appropriate space under a corresponding transform, the bounded variation space in this case. This leads to clear benefits in all the cases where few measurements are available or are characterised by strong undersampling [\[117\]](#). In addition, the [TV](#) algorithm is able to produce solutions with clear background and sharp edges [\[134, 135\]](#). The drawback associated to the [TV](#) regularisation is linked to the computational complexity introduced by the l_1 norm which is a non differentiable term and is not rotationally invariant, which might result in geometric distortions in the directions of the coordinate axes. A modification of the [TV](#) regularisation can be also used, in which a l_2 norm is forced in the [TV](#) term, called isotropic [TV](#) regularisation. However, this approach has proven to be less strong than its l_1 formulation [\[136\]](#). The consequence of the use of the [TV](#) is that a piecewise constant solution is searched by the algorithm as it is an a-priori information promoted by the regularisation term. This constitutes a limitation in the case of biological samples which are generally characterised by a smooth profile, rather than a piecewise constant one. This aspect will be in-depth discussed in Chapter 3 of this elaborate.

2.1.4.2 Mathematical solution of the TV minimisation

The solution of Eq. (2.46) is non-trivial due to the presence of the non differentiable [TV](#) term. Many approaches have been proposed in the literature to handle this minimisation, however, in this elaborate we will resort to the one proposed by [\[137\]](#) as it proven to be the state-of-the-art solver in terms of allowed performance. Eq. (2.46) can be rewritten

according to

$$\min_x \|D_i(x)\|_1 + \frac{\mu}{2} \|Ax - b\|_2^2 \quad (2.47)$$

In Eq. (2.47), $x \in R^{s \times t}$, with $s \times t = n$, represents an image, $D_i(x) \in R^2$ represents the discrete gradient of x at pixel i , $A \in R^{m \times n}$ represents the measurement matrix, $b \in R^m$ is the measurement of x . To separate the non-differentiable l_1 norm term, a variable splitting can be introduced introducing $y_i = D_i x$. Eq. (2.47) is therefore written as

$$\min_{y_i, x} \|y_i\|_1 + \frac{\mu}{2} \|Ax - b\|_2^2 \quad s.t. \ D_i x = y_i \forall i \quad (2.48)$$

Which represents the equation we need to minimise. Eq. (2.48) can be regarded as a special form of the equality constrained minimisation problem in Eq. (2.49)

$$\min_{y, x} f(x, y) \quad s.t. \ h(x, y) = 0 \quad (2.49)$$

In which $x \in R^{n_1}$, $y \in R^{n_2}$, $h : R^{n_1+n_2} \rightarrow R^m$ are differentiable with respect to both x and y but the function f might or not be differentiable with respect to y .

The minimisation of the Eq. (2.49) in the form of Eq. (2.48) is made in [137] following a procedure based over the minimisation of the Augmented Lagrangian function called Augmented Lagrangian Method (ALM). In ALM, first suggested by Hestenes [138] and Powell [139], the lagrangian multipliers are introduced and adjusted at every iteration. The augmented lagrangian equation for Eq. (2.49) can be expressed in general form as Eq. (2.50), where the "T" denotes the transpose operator and λ the lagrangian multiplier.

$$L_A(x, y, \lambda; \mu) = f(x, y) - \lambda^T h(x, y) + \frac{\mu}{2} h(x, y)^T h(x, y), \mu > 0 \quad (2.50)$$

According to the ALM framework, the k -th iterate (x^k, y^k) is obtained minimising Eq. (2.50) jointly with respect to both x and y for a given multiplier estimate $\lambda = \lambda_{k-1}$, then the multiplier is updated according to $\lambda_k = \lambda_{k-1} - \mu h(x_k, y_k)$. The parameter μ is called the penalty operator of the ALM equation. Eq. (2.50) is particularised in the case of Eq. (2.48)

as

$$\phi(x, y) = L_A(x, y, \nu, \lambda; \beta, \mu) = \sum_i [\|y_i\|_p - \nu_i^T(D_i x - y_i) + \frac{\beta}{2}\|D_i x - y_i\|_2^2] + \frac{\mu}{2}\|Ax - b - \frac{\lambda}{\mu}\|_2^2 \quad (2.51)$$

The complexity of the minimisation problem in Eq. (2.49) is therefore entirely shifted over the minimisation of Eq. (2.50) jointly with respect to x and y . Therefore, the need is to minimise an unconstrained problem as in Eq. (2.52)

$$\min_{y, x} f(x, y) \quad (2.52)$$

This can be made efficiently considering some little-restrictive hypotheses over Eq. (2.52) which regard the required complexity for the joint minimisation and are in practice very common. In fact, supposing the so-called uneven complexity structure, if the complexity of the minimisation regarding one of the two variables is much lower than the other, interesting structures can be used to minimise the equation efficiently. Once the hypothesis of uneven complexity is forced, with only a little loss of generality Eq. (2.52) assumes a form as in (2.53),

$$\min_x \psi(x) := \phi(x, y(x)) \quad (2.53)$$

transforming the joint constrained minimisation of Eq. (2.49) in an unconstrained minimisation problem with respect to one variable as in Eq. (2.53). For the minimisation of Eq. (2.53), the authors refer to an adaptation of the non-monotone line search algorithm [140] proposed by Zhang and Hager to non-differentiable object functions for which only the existence of one partial derivative with respect to x is required. Non-monotone line search algorithm is used in conjunction with another algorithmic idea which is the Alternating Direction Method (ADM). This method, proposed by [141, 142], requires not to minimise the function Eq. (2.50) at every iteration jointly with respect to both the variables, but only with respect to x and y respectively before updating the multipliers. Using these two algorithmic structures, the authors in [137] develop an algorithm for minimising TV norm with great efficiency, for which ADM method enables taking full advantages of the low-cost minimisation in the “easy” direction,

and the non-monotone line search allows relatively quick and large steps in the “hard” direction. The authors introduce for it the name NADA, Non-monotone Alternating Airection Algorithm. NADA is applied at every iteration of the minimisation of Eq. (2.51). The resulting numerical solver which they propose is called TVAL3 [137] (Total Variation Augmented Lagrangian ALternating-direction ALgorithm) for solving Eq. (2.49) whose pseudocode is presented below in Algorithm 1.

Algorithm 1 Total Variation Augmented Lagrangian ALternating-direction ALgorithm

Input: Initialize (x_0, y_0) , $\mu_0 > 0$, $\lambda_0 = 0 \in R^m$

Input: Set $k = 0$

Output: (x_{k+1}, y_{k+1})

while *Not Converged* **do**

- (1) Call NADA $\rightarrow$ Minimise $\phi(x, y) := L_A(x, y, \lambda_k; \mu_k)$
- (2) Update the multiplier $\rightarrow \lambda_{k+1} = \lambda_k - \mu_k h(x_{k+1}, y_{k+1})$
- (3) If necessary, update the penalty parameter to get μ_{k+1}
- (4) Increment k value $\rightarrow k = k + 1$

end

2.2 Tomographic Phase Microscopy

As mentioned above in Eq. (1.1), QPMs contain an integral coupled information about the OPD of light propagating into the sample. Decoupling the information about the thickness of the sample and its RI is necessary to get access to the 3D information about the internal morphology of the sample. To do that, tomography is employed. If QPMs corresponding to different orientations are available, then with the described approaches it is possible to recover the information of interest. In order to obtain QPMs relative to different orientations, three configurations are generally employed, sketched in order in Fig. 2.6 [143]: (i) Illumination Scanning Configuration (ISC), (ii) sample/mechanical/optical rotation and (iii) in-flow Sample Rotation Configuration (SRC) in microfluidic environment. (i) requires fixing the position of the sample and scanning the illumination around it [121, 144]. However, during the scanning, no further interaction

with the sample is needed and the illumination angles are finely controlled with a precision that can go below the degree.

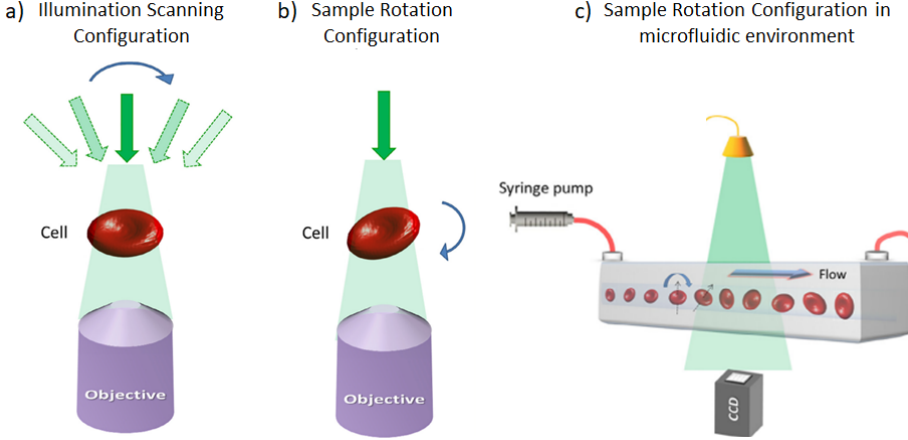


Figure 2.6. Different configurations for TPM. (a) ISC. (b) SRC. (c) SRC in a microfluidic environment. Images reproduced from [143].

The technologies allowing the illumination scan define different groups of ISC systems. Systems as in [121, 145, 146] use a galvanometric mirror to scan the beam. In [147], [148], [149], spatial light modulators are used, whereas in [150] a digital micromirror is mounted. Recently, in [151, 42] white light sources have been employed for an enhanced resolution of the reconstructed tomograms. In [144, 152, 153] the possibility to employ a sparse angle illumination is discussed, whereas in [154, 155, 156, 157] a lens-free holographic microscope was proposed to perform the imaging of a large FOV incorporating super resolution techniques in the tomographic reconstruction. Even though ISC configuration is commonly employed, it introduces important drawbacks which must be accounted for. Given the need to physically place the sample in the correct position to be probed, the throughput of the acquisition is low. These system also require careful and delicate alignments [146, 121]. In addition, the illumination range generally is angularly limited, ranging from -70 degrees to 70 degrees, owing to the fact that the detector is stationary during the measurement process and the illumination is rotating, determining the limited angular range of illumination directions within which the projections can be acquired. This

results in a peculiar problem during the reconstruction phase called missing cone problem, introduced in the previous chapters. The latter arises from the finite Numerical Aperture (NA) of an optical system which limits the fraction of diffracted light coming from the sample which can be probed by the detectors [118]. This determines an incomplete coverage of the frequency components causing problems in the tomographic inversion, as sketched in Fig. 2.7. Indeed, severe artefacts in the reconstructions are introduced, in particular in the direction of the optical axis, and wrong estimates of the RI of the sample that must be handled numerically. Systems as in (ii) allow to change the relative position of the light and the sample by fixing the position of the light and rotating the sample with a direct manipulation. This can be accomplished mechanically [158] or employing systems such as holographic optical tweezers [148, 159, 160, 161]. The drawback in the use of these configuration is linked to the low throughput they offer and the risk to alter the sample during the manipulation and to the enormous complications they comport in the optical setup. Finally, configuration as in (iii) represent a flexible solution which is lately gaining prominent attention. To surpass the limited throughput of the aforementioned systems, the tomographic microscopy principle has been implemented and demonstrated in microfluidic condition. For example, it can be induced by placing cavities in the channel to attract and trap the particles and make them rotate thanks to the turbulence created [162]. The introduction and removal of particles into and from the cavities is done by optical traps. Other approaches exploit a shear flow [163, 164, 165] to induce the rotation. In these systems the particles rotate due to the effect of the velocity gradient present into the channel which acts over the sample, as it was demonstrated for the first time by [73] in a microfluidic condition. Hence, in SRC given the lack of need of physically manipulate the sample to rotate it, elevate throughput can be reached. In addition, an isotropic illumination of the sample is feasible, overcoming the problem of the limited angle. Finally, as no interaction with the sample is needed, the risk of damaging it is lowered. These advantages make this technique easily employable in in-flow imaging and analysis applications which are well established in the field of biomedical imaging, as it will be thoroughly discussed in the next chapters. However, SRC comports the additional complexity of not allowing to finely control the rotation angle,

which must be numerically recovered a-posteriori, potentially introducing errors into the reconstruction process. Moreover, a precise control of the flux in the channel is still not available, leading to non-complete control of the rotation of the sample in terms of angular speed. Anyway, the flux can be engineered to address this issue [166, 167] therefore a great progress in these systems is expected.

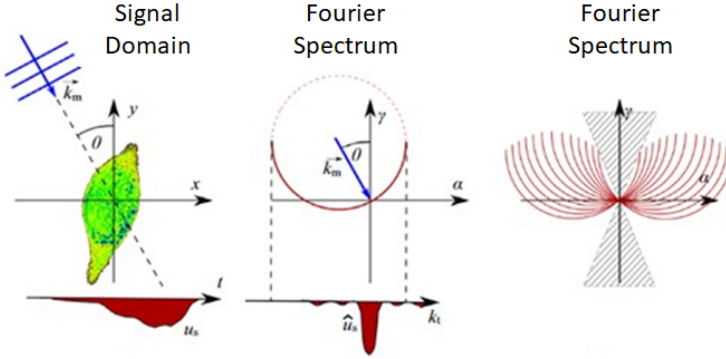


Figure 2.7. Illustration of the origin of the problem of missing cone for Limited Angle Tomography (LAT) ODT. The angularly deficient sampling of the sample results in a loss of frequential information and an incomplete filling of the Fourier space. Images reproduced from reference [168].

2.2.1 Applications of TPM to biological imaging

As mentioned in the previous chapters, TPM allows to fully extract the 3D information conveyed by DH data, opening up new directions of investigation. In particular, it allows to reconstruct the morphological inner structure of a biological sample and all its internal substructures without the need of any external labelling agent. This gives access to a complete new set of measurable morphological parameters that carry insightful biological information. Its popularity in the biomedical community is quickly increasing mainly due to the availability of the commercial holographic tomography systems offered by the companies Tomocube Inc. [169] and Nanolive [170]. As reported in [1], through TPM the shape and dry masses of condensed chromosomes of HT-29 and T84 cells in metaphase were mea-

sured [122]; in addition, internal particles such as lipids, gold nanoparticles or SiO₂ microspheres can be clearly identified due to highly distinctive RI values and thus used to mark samples in a label free manner [171]. The morphology and internal structures of yeast and bacteria have been investigated too [172, 173]. Recently, label-free tomography has been extended to optically thick specimens, such as embryos or brain slices, which traditionally due to excessive scattering were imaged through different techniques. Moreover, since the use of QPI has proven to be a powerful approach for quantifying the efficacy of cell immunotherapy [1, 174] with interesting applicative potentialities as screening and monitoring procedure, TPM has been used to obtain yet more information. As the morphologies of live cells are significantly altered by disease states such as viral infection and cancer, TPM constitutes a powerful tool to visualise them for long periods of time as it does not require fixation and labelling of the samples [37, 175]. By measuring cell thickness using TPM, phenotyping of cancer cells was demonstrated [176] or used to discriminate between different cancer types [177]. Quantitative and label-free imaging capability makes TPM an effective method for blood screening. One of its most common applications regard the study of RBC, as their morphology is directly linked to their health status which correlates to many systemic diseases such as diabetes, thrombosis and malaria [152, 153, 178]. Through TPM of RBC the systematic and correlative analysis of various cellular parameters, such as morphological (cell volume, cell surface area, sphericity index), biochemical (haemoglobin concentration, haemoglobin contents and biomechanical) and cell membrane deformability at the individual cell level can be measured quantitatively. TPM has also been applied to the study of white blood cells [153], neuron cells [178], hepatocytes [13]. Also, TPM provides label-free sperm analysis, which can be potentially used in in-vitro fertilisation [179] or to study male infertility as the motility and morphology of sperm cells can be measured [160].

2.3 TPM in flow cytometry mode

As mentioned above, TPM is a well-established technique which allows to fully exploit the information conveyed by digital holograms. Over the last years TPM devices have successfully entered the market, such as the

NanoLive inc [170] and TomoCube [169] and have been used for biological investigation. Both these products allow to obtain label free, 3D reconstruction of biological samples by the means of TPM-ISC mode, and the possibility to include a non coherent illumination to increase the resolution is being explored [180], [181]. Nowadays, however, the greatest limitation of TPM is the throughput it offers, which is significantly reduced if compared to FC and IFC devices [113]. This issue depends on the nature of the tomographic imaging itself and on the configuration used to perform it. Indeed, the developed systems operate in the ISC which slows down the acquisition. Moreover, many acquisitions for each sample are needed for tomography, additionally slowing down the process. In order to fully exploit the advantages of this technique, different solution must be explored. In particular, in 2017 the paradigm of TPIFC has been demonstrated for the first time at ISASI-CNR. In in-flow modality, the QPMs are acquired as the sample flows and rotates into a microfluidic channel, thus allowing an elevate throughput. Therefore, TPIFC is the technology that can best conjugate the advantages of TPM and FC.

2.3.1 TPIFC setup at ISASI-CNR

At ISASI-CNR the state-of-the-art TPIFC is realized. The setup is based on a DH microscope mounted in an off-axis configuration using the Mach-Zehnder interferometric scheme as illustrated in Fig. 2.8 (a). The coherent illumination source is a laser light (Laser quantum torus 532) which emits light in the green spectrum at 532 nm with an output power of 750 mW. A Polarised Beam Splitter (PBS) splits the coherent light into the reference and the object beam. Two half-wave plates are placed in front of and behind the PBS, to balance the ratio between the intensity of the two beams without altering their polarisation. The object beam is directed towards the sample which flows into a microfluidic chip (Microfluidic ChipShop 10000107 - $200\text{ }\mu\text{m} \times 1000\text{ mm} \times 58.5\text{ mm}$). The object-modulated emerging wavefront is collected by a MO (MO1, Zeiss Plan-Apochromat, 40 $\times$, NA=1.3, Oil immersion) and sent to a tube lens (TL1 with focal length 150 mm). The reference beam is instead expanded by a beam expander with a MO and a tube lens (MO2, Newport, 20 $\times$, NA=0.40) and a second tube lens (TL2 with focal length 250 mm). A Beam Splitter (BSP) is used to combine the object and reference beam

at a slight angle to obtain off-axis holography. The resulting interference pattern is digitally recorded by a CMOS camera (Genie Nano-CXP Cameras, 5120×5120 pixels and 4.5 μm pixel size) characterised by a FOV of 640×640 μm^2 and a spatial resolution of 0.5 μm . The camera is used in video recording configuration with a long-time acquisition mode and records samples along the z optical dimension that flow and rotate in the y direction with a framerate of 35 fps.

2.3.2 Strategies for inducing cells rotation in the microfluidic channel

The rotation of the samples in the channel is obtained according to the principles of hydrodynamics. Indeed, a laminar flow ($\approx 50\text{nL/s}$) is generated by an automatic syringe pump (Syringe Pump neMESYS 290N), which generates a momentum over the sample, inducing its rotation. The pump is a low-pressure system which allows a high precision and pulsation-free dosing of liquids at micro and nanolitre scale ensuring a very homogeneous flow inside the microchannel. The volumetric flow rate Q is related to the pressure drop δP in a fluid following through a long pipe by the Hagen Poiseuille Eq. $\delta P = R_{hyd}dQ$. In this expression, $\delta P = R_{hyd}$ is the hydraulic resistance of the channel, supposing that the fluid is viscous and incompressible and a laminar flow exists, conditions that are always met in microfluidics [182]. Eq. (2.54) developed in [167]

$$v(x, z) = \frac{16h^3}{\mu\pi^3} \frac{-dp}{dy} \sum_{i=1}^{+\infty} (-1)^{\frac{i-1}{2}} \left[1 - \frac{\cosh(\frac{i\pi z}{2h})}{\cosh(\frac{i\pi w}{2h})} \right] \frac{\cos(\frac{i\pi x}{2h})}{i^3} \quad (2.54)$$

expresses the flow velocity in the channel with a rectangular cross-section, identifying a parabolic profile which is maximum at its centre and minimum around the borders. In Eq. (2.54), $\frac{dp}{dx}$ is the pressure drop across the channel, and h and w are the height and the width of the channel cross-section x, z , respectively, as depicted in Fig. 2.8. As the cells are introduced in the microfluidic channel, under effect of gravity they accumulate over the bottom surface of the channel. According to Eq. (2.54),

the sides of the cells facing the central part of the channel and the bottom surface will be exposed to different velocity profiles. Therefore, a torque is generated over the sample which is put into rotation. As the cell flows into the channel and the rotation is induced as described above, holograms corresponding to the different orientations are acquired keeping the illumination in a fixed position. The advantage of this tomographic configuration lays also in the limitation of the limited angle issue, which generally affects [TPM in ISC](#) acquisition as an incomplete angular coverage is reached. Instead, in the in-flow configuration the angular coverage is complete around one axis. Moreover, the biological sample is not altered by the mechanical/optical external forces needed to rotate it, as would happen in system employing other techniques to induce its rotation [148]. Furthermore, as the hydrodynamics-based rotation is applied to all the cells which enter into the microfluidic device, the throughput of the tomographic system can be even more increased. Finally, as the cells are not stitched to a physical support such as a petri dish, their dynamic rotational behaviour can be observed. This is particularly interesting for the case of the samples which naturally live in an in-flow condition, such as blood cells and [CTCs](#), which therefore are observed in their natural condition. An example of recorded digital hologram is displayed in Fig. 2.8 (c), while a sequence of successive holograms cut in the same vertical region is reported Fig. 2.8 (d) to show the cell flow along the y-axis.

2.3.3 Speeding up the reconstruction process for TPIFC

The [DH](#) reconstruction process described in Chapter 1, even though automatic, might constitute a bottleneck in the case of the desired real-time tomographic reconstruction. For tomography, in general, a great number of projections is sought after to obtain a reconstruction of good quality. However, in this eventuality, the holographic reconstruction process becomes time consuming in particular in [LOC](#) applications where the hardware resources are limited. Depending on the hologram size (i.e., the number of pixels of the sensor camera) and the imaging configuration (i.e., in-line or off-axis), the entire processing pipeline to reconstruct one single [QPM](#) from the recorded digital hologram can take minutes on a basic desktop computer. When hundreds of [QPMs](#) must be reconstructed the time needed to produce a single tomogram might become prohibitive. Many approaches

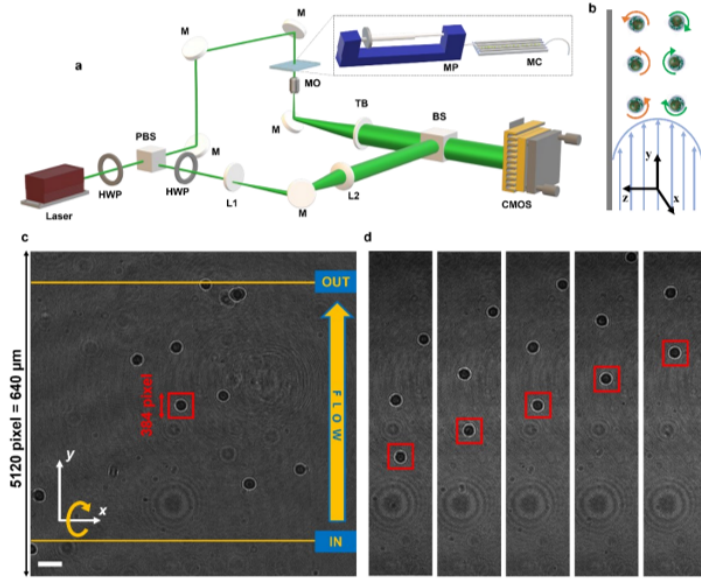


Figure 2.8. Sketch of the TPIFC system developed at ISASI-CNR, (a) PBS. WP - Wave Plate. M - Mirror. L1-L2 - Lens. MO - Microscope Objective. MC - Microfluidic Channel. MP - Microfluidic Pump. TL - Tube Lens. Batch Size (BS). (b) Sketch of the SRC system in microfluidic environment, in which the cells flow and rotate due to the hydrodynamic forces generated into the channel. The reference system in use is indicated. (c) FOV of the camera with different cells appearing. (d) Succession of digital holograms acquired for a single cell flowing in the FOV highlighting the movement of the cell. Scale bar = $50 \mu m$.

have been proposed in the literature to address this issue using data driven methods [183]. These methods are particular suitable for IFC systems and in particular TPIFC systems, as the throughput of IFC-based methods allow to acquire a great number of images in a short time and therefore make it easy to build large datasets. In addition, they are often convenient respect standard physics-driven approaches where many parameters have to be tuned often experimentally. Data driven approaches have been used at different stages of the reconstruction pipeline. Many groups propose to use them to accelerate the phase of numerical refocusing [184, 185], which is time consuming since it needs to sweep along the entire set of available focusing distances to find the correct one [184, 186, 187, 188]. Other researchers used them to compensate the phase aberrations [189], or to suppress the twin image [190]. Deep Convolutional Neural Network (DCNN)s inspired by the ResNet model have been employed for both the automatic phase aberration compensation [191] and phase unwrapping process, which is another particularly time-consuming step of the procedure [192, 193, 194]. Many others use data driven approaches to accelerate the denoising process of the QPMs [195, 196, 197, 198, 199, 200] or to increase the resolution of the acquired holograms or the reconstructed QPMs, which is limited according to the finite pixel size of the camera [201, 202, 203]. Finally, researches have successfully demonstrated the use of data driven approaches for the entire reconstruction process from the digital hologram to the QPMs [190, 204, 205, 206, 207]. In particular, in [208] this problem was solved with the idea of going towards real time, LOC application. Indeed, a particularly lightweight network was used, the CAN, which given the reduced numbers of trainable parameters allow a really fast inference with a weight occupation compatible with commercial microcontrollers. In particular, considering a Intel Core i9- 9900K CPU with a 64Gb RAM through the Matlab 2021a environment, the process of QPMs reconstruction was sped up from 7.71 s to 168 ms, with a memory occupation of 818 kB, see [208].

2.3.4 A-posteriori rolling angle recovery in TPM-SRC

To proceed with the tomographic reconstruction, the integral measurement data must be associated to a tomographic angle corresponding to that specific projection. As mentioned above, the tomographic paradigm

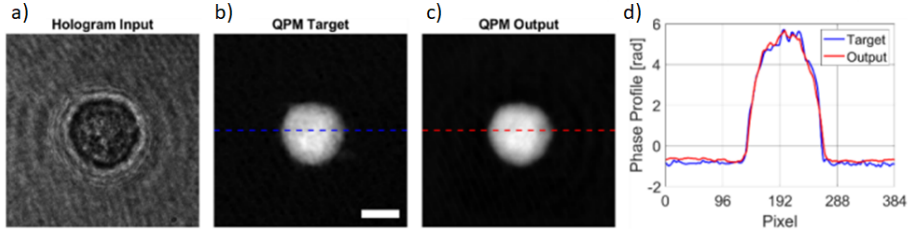


Figure 2.9. CAN CNN ability to replicate the entire holographic processing pipeline. (a) Input hologram. (b) Target QPM. (c) CAN - generated QPM. (d) Horizontal cut highlighting the quality of the reconstruction. Image replicated from [208].

in use at ISASI-CNR is the one of sample rotation. As a consequence, no a-priori information is available over the tomographic angles which are not predetermined, therefore they constitute an unknown of the problem and must be recovered a-posteriori. Several approaches have been proposed and successfully applied at ISASI-CNR for this problem. In [73], an approach specific for RBC was proposed, linked to the principle that each of them can be modelled as microlens given their shape and quasi-homogeneous RI distribution [209]. Accordingly, a mathematical relation can be found between the rotational angles and the variation of the Zernike coefficients during the cell rotation. Another approach was developed in [73] for cells having high RI variations and strongly non symmetrical shapes. In this case the rolling angles are estimated according to the maximisation of the correlation coefficient between pairs of mirrored phase images for which the angles sum is 180° . This method is however non applicable to specimens with elevate isotropy as the identification of the couple of specular images is based on symmetric consideration. Many biological samples unfortunately fall into this category, such as blood cells or CTCs. Accordingly, another approach was developed in [210] for samples with quasi-spherical symmetry where the angular sequence is reconstructed exploiting information obtained through a theoretical microfluidic modelling of cell rotation and relative centroids movement. Even though successful, this method is computationally expensive and therefore slow given the need to evaluate for each frame a complex mathematical model, and also strongly dependent on the noise level. The need to develop a more general and easy approach

was solved in [211]. The authors proposed a method based on the identification of the frame corresponding to a half or a full rotational event, such as the 180 or 360 degrees projection, and then the tomographic angular sequence is reconstructed supposing a proportionality between the rotation and the translation of the cells into the microfluidic channel. The validity of this hypothesis directly depends on the properties of the microfluidic environment which was accurately discussed in [211] but can be generally regarded as true. The first procedure of the method consists in assigning the value of 0 degrees to the first frame, which can be made without loss of generality. Then, the QPM associated to a half rotational projection is identified as the one which minimises the Tamura Similarity Index (TSI) [211] evaluated for all the frames available. Let $QPM(k)$ be the phase reconstruction of the k -th frame with sizes $N \times M$, and let $S_{i,j}(k)$ a 3×3 patch within $QPM(k)$, centred in the pixel of coordinates (i, j) , with $i = 2, \dots, N - 1$ and $j = 2, \dots, M - 1$. The values of $S_{i,j}(k)$ are assigned as the TC calculated over the patch $S_{i,j}(k)$ and allow to obtain a new image of sizes $(N - 2) \times (M - 2)$ called the Local Contrast Image (LCI), whose generic element is

$$LCI_{i,j}(k) = TC(S_{i,j}(k)) \quad (2.55)$$

with $i = 2, \dots, N - 1$, $j = 2, \dots, M - 1$ and $k = 1, \dots, K$. Finally, the TSI image is evaluated as

$$TSI(k) = TC\left(\frac{LCI(1)}{flip(LCI(k))}\right) \quad (2.56)$$

where the flip operator represents the horizontal mirroring respect to the x axis. TSI in Eq. (2.56) denotes a convex functional where the global minima represents the index of the 180th image.

$$f_{180} = argmin_k(TSI(k)) \quad (2.57)$$

Once the 180th image is found, the entire angular sequence can be retrieved according to

$$\theta_k = 180 \frac{y_k - y_1}{y_{f_{180}} - y_1} \quad (2.58)$$

The described procedure can be adapted to the search of the 360 frame removing the flip from the formula. Generally, the TSI metric exhibits a

great stability in the search of the 180 image index, being less prone to be influenced by the noise present over the QPM and therefore presenting a smoother profile and a minor number of local minima, as was studied by the authors of [211] comparing with the most common used similarity metrics (Spatial Correlation Coefficient (SCC) [73], Structural Similarity Index Matrix (SSIM) [212], Universal Image Quality Index (UIQI) [213], Gradient Magnitude Similarity Deviation (GMSD) [214]). Several considerations must be made regarding the described rolling angle recovery procedure. The entire reconstruction process is dependent over the exactness of the assignation of the 180th frame. An error in the estimation directly propagates over the entire reconstructed sequence and the tolerance of the reconstruction algorithms towards this phenomenon is limited, as highlighted in [211]. The error in the estimation of the 180 frame might depend on the level of noise of the QPM or by a physical lack of that specific projection angle, given the finite framerate of the camera and the speed of the cell in the channel which is not completely automatised and therefore controlled. Moreover, there is a trade off between the speed of the cell and the throughput of the tomographic technique. Some applications where a greater throughput is sought after result in a greater angular step between rotations and therefore the loss of the full rotational event. Generally, a setup as the one introduced in this Chapter results in several hundreds of projections available in total with multiple full rotations for a single specimen. The 360 rotation event is generally estimated around the 100th projection and the angular step of the retrieved sequence is in the order of the 2-3 degrees.

Chapter 3

Towards Lab-on-chip TPIFC

As previously discussed, standard experimental realisations of off-axis [DH](#) are mostly based on the Mach–Zehnder interferometric architecture. Even though this configuration is extremely used, it is not the ideal one to rely on with the perspective of advancing towards applications for outside of optical labs, in particular for [LOC](#) solutions. Indeed, [LOC](#) devices are meant to be compact and portable; moreover, they should be easy to handle by common people for everyday life usage. Finally, they should be cheap enough to promote their large-scale diffusion. [TPIFC](#) setups based on a Mach–Zehnder architecture are often bulky, intended for use in optical laboratories. This configuration requires sophisticated, generally expensive hardware equipment which raises up the price of the total setup and limits its use to specifically trained technicians. However, the main disadvantage of Mach–Zehnder based optical systems is linked to the interferometric configuration itself. Indeed, the two optical paths of the mutually coherent signal and reference waves that provide the interference record are completely separated. As a consequence, the two beams face uncorrelated disturbances that are not cancelled when the interference map is recorded and constitute an additional noise contribution over the hologram that must be dealt with. Also, this constitutes a problem in terms of loss of temporal stability of the acquired interferograms with serious consequences over the phase information. Therefore, this approach exhibits high sensitivity to mechanical vibrations and environmental noise that make Mach-Zehnder based setups in practice unfeasible for applications outside

of optical labs. To overcome these issues, common path approaches have been successfully proposed in the literature and effectively used to go towards [LOC](#) oriented solution [215, 216]. However, the resulting setups often exhibit a poor optical performance which results in a poorer quality of the acquired data. This has to be handled by incorporating more robust numerical reconstruction approaches allowing to recuperate for the hardware-linked loss. This paradigm, called Computational Tomography, will be object of the discussion of the next chapters of this work.

In this Chapter, the common path setup developed at ISASI-CNR will be described, with a focus on the criticises it poses. To deal with these more challenging conditions, a robust numerical approach, the [HOTV](#), is used for the first time in [TPIFC](#) for the reconstruction of experimental data. Finally, the possibility to incorporate fractional calculus into this numerical technique will be introduced as an open research issue.

3.1 Common path interferometric setups

Common path setups were introduced to solve the problems of loss of mutual coherence between the two branches [215, 216]. The idea behind them is that the two beams involved in the holographic process share identical or similar optical paths, allowing to compensate for environmental vibrations, as they face correlated disturbance than can get easily cancelled. Consequently, these setups show a significantly enhanced temporal stability. In addition, the compact and robust common path configuration is advantageous in optical instrument manufacturing, which makes them particularly suitable for [LOC](#) applications. A key issue in designing common path configurations is the generation of a sufficiently uniform reference beam. The solutions proposed to this end allow to categorise common path configurations into two main categories, namely lateral shearing or self-referencing and point diffraction models. In lateral shearing setups, the principle of shearing interferometry is used. The beam is first doubled using a designated optical component. The two resulting replicas are therefore laterally separated, this separation is referred to as shear. The overlapping region of the two displaced beams is used to form the hologram, which is collected in an off-axis configuration if an angular tilt is introduced for one of the two branches. The term self-referencing is used

as the reference beam is not separately generated but is obtained as a sample-free portion of the object wave. An example of lateral shearing common path setup is shown in Fig. 3.1 (a). In this configuration, as the

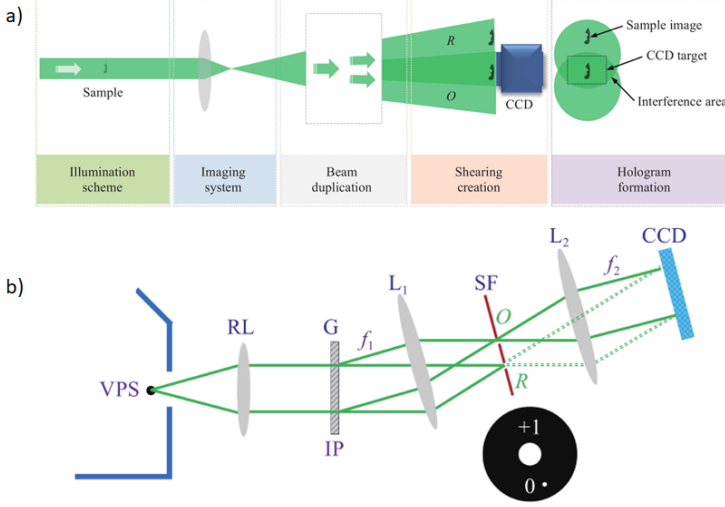


Figure 3.1. Common path setups main configurations. (a) Lateral-shearing common path setup. (b) Point diffraction common path setup. VPS -Virtual Point Source. RL - Relay Lens. G - Grating. IP = Image Plane. L1-2 - Lenses. SF - Spatial Filter. Images replicated from reference [215].

beam is entirely duplicated, both the beams carry the same information. Therefore, the reference beam is constituted by a sample-free region in one of the two replicas. As a consequence, this configuration is suitable for bounded or sparse samples. Moreover, the shearing distance must be big enough so that only one of the two replicas is sent to the camera. Note that the second replica could be used to enhance the resolution of the reconstruction as in [217]. Regarding the components used to duplicate the beam, multiple choices are available. Among others, glass plates [218, 219, 220, 221] and BSP [222, 223, 224] are typically employed. Concerning glass plates, these optical components allow to duplicate the beam as the incident field is reflected at the front and at the back of the device when it is oriented at approximately 45 degrees with respect to the optical axis of the incident beam. The advantage of this configuration is that it can be easily integrated within a preexisting commercial microscope. As

mentioned, [BSP](#) can also be employed to construct lateral shearing-based common path [DHM](#) configurations. The output beam from a microscopic imaging system contains the half-object and half-reference parts. Then, the entire beam is further divided by the [BSP](#) to create the sheared beams. Finally, the half-object and half-reference beams interfere with each other at a certain angle [215]. Other optical components such as beam displacers, Wollaston prisms, Rochon polarisers, gradient-index lenses, and biprisms can also be utilised to build the shearing device.

Common path configuration based on point diffraction are used to overcome the drawbacks of the lateral shearing configurations regarding the presence of a duplicated image and allow to lift the hypothesis of imaging of sparse-only samples. In these setups the object and reference beams are not laterally displaced but share the exact same optical path. The reference beam is obtained filtering the sample-linked information from the object wave, generally exploiting gratings. Fig. 3.1 (b) [21] illustrates an example of point-diffraction common path setup, where a magnified sample image is obtained in an inverted microscope configuration. To illustrate the imaging process, the starting point is a virtual point source (VPS) representing the object plane. At the image plane (IP), a grating G replicates the microscopic image and generates several diffraction orders. Then, these orders enter a $4f$ unit with lenses $L1$ and $L2$. The 0th order beam is low pass filtered by a pinhole at the focal plane of $L1$, resulting in a uniform reference beam. Simultaneously, one of the 1st order beams containing complete sample information passes through the unit and becomes the object beam. Finally, the two beams interfere with each other on the CCD target at an appropriate off-axis angle. As this common path configuration generates the uniform reference beam by diffracting the object beam through a “point” pinhole, this model is referred to as “point diffraction model”. Other configurations can be found in [215].

3.1.1 Common path setup for TPM

For the first time in [225], at the ISASI-CNR labs an experimental layout based on a quasi-common path lateral-shearing architecture was successfully integrated into the [TPIFC](#) reconstruction of cells. In addition, the numerical reconstruction process was adapted to the new acquired data to allow the accurate retrieval of 3D tomograms of experimental data

consisting of White Blood Cell (WBC) from the line THP-1 and mouse fibroblasts from the line NIH-3T3. For the first time in TPIFC, the tomographic data are reconstructed resorting to a high-order version of the TV technique described in section 2.1.4.1. Moreover, a new experimental strategy for testing the system's reliability by assessing the repeatability of the digital holographic tomography apparatus is discussed. The setup was designed with the idea of advancing towards portable applications. Therefore, for the configuration, cheaper and more easy-to-use components were employed. In particular, a laser-light illumination (Sapphire SF, $\lambda = 488nm$), a long-working-distance objective (MO; Nikon, $20\times/0.50$), and a camera with a relatively small sensor area and a lower recording frame rate (CMOS; UI-3370CP-M-GL, 2048×2048 , $5.5 \mu m$ square pixels) were selected. Initially, the linearly polarized laser beam is spatially filtered via focusing and collimation optics and directed toward the sample. Light scattered by the sample is collected by the MO, which shapes the passing-through light; hence, the image arises in proximity to the camera-chip plane. The investigated sample consists of cells flowing inside the microfluidic channel. The whole in-flow path begins with a syringe pump, including the input reservoir with cells, polymer tubing paths, microfluidic chip (MC, Straight 4-channel Mini-Luer Chip (P/N 10000091)), and output tubing with a waste reservoir. The chosen common path configuration is based on a Michelson interferometer and relies on a Shearing Device (SD) to create the interferogram. Here, the SD consists of a BSP and two mirrors M2 and M3; which provide the two duplicated images of the sample of interest. The first image arises from the optical path $BS \rightarrow M2 \rightarrow BS$, and the second arises following the path $BS \rightarrow M3 \rightarrow BS$. The different tilts of the mirrors M2 and M3 allow to create an angular tilt and lateral displacement of the two replicas, allowing off-axis digital holography. The two replicas are directed towards the camera. A portion of the observed field sketched in Fig. 3.2, area 1, serves as a signal beam, while the sample-free area in the replica sketched in Fig. 3.2, area 2, constitutes the reference beam. In the present experimental configuration, the lateral shift among both replicated images is comparable to the dimensions of the used CMOS chip, so only one of the replicas is collected by the camera. The Iris diaphragm (I), inserted between the MO and CMOS, reduces the amount of unwanted back-reflections and stray light. The lateral

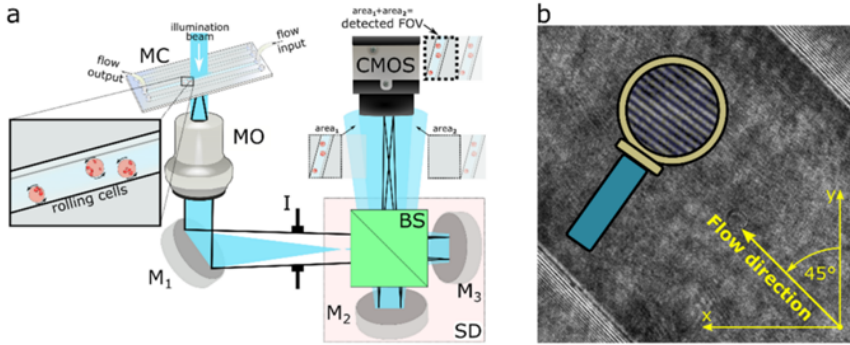


Figure 3.2. Common path setup developed at ISASI-CNR. (a) Sketch of the setup. MC- Microfluidic Chip. MO - Microscope Objective. SD - Shearing Device. BS - Beam Splitter. M1-2-3 - Mirrors. I - Iris diagram. (b) Visualization of the acquired digital hologram with an enlarged detailing of the interference fringes. The angularly tilted flow direction is highlighted compared to the introduced reference system. Image replicated from [225].

magnification of the imaging system, measured by a positive USAF 1951 amplitude line target, was established as 55, and the expected theoretical lateral resolution in the object space can be approximated as $0.82\lambda/\text{NA} \simeq 0.8 \mu\text{m}$. In order to recuperate as much FOV as possible, a 45 degree tilt between the camera and the microfluidic direction is performed. Finally, the term quasi-common path architecture reminds that there is a slight separation in the optical paths of the two beams between the BS and the mirrors (paths $\text{BS} \rightarrow M_2 \rightarrow \text{BS}$ and $\text{BS} \rightarrow M_3 \rightarrow \text{BS}$) due to the choice of the Michelson-based SD. This might introduce a loss in the mutual coherence of the two beams. However, the effect is considerably smaller if compared to the Mach-Zehnder configuration, in particular if a source with a sufficient temporal and spatial coherence is chosen. Totally common path architectures are feasible and constitute the undergoing development of the proposed setup, exploiting a Sagnac interferometric architecture to obtain a completely overlapping optical paths for the beams, as shown in Fig. 3.3, where the two SDs based respectively on the Michelson geometry and on the Sagnac one are compared. In the latter, the SD consists of a BSP and mirrors M_1 , M_2 , M_3 and the initial beam is split into two

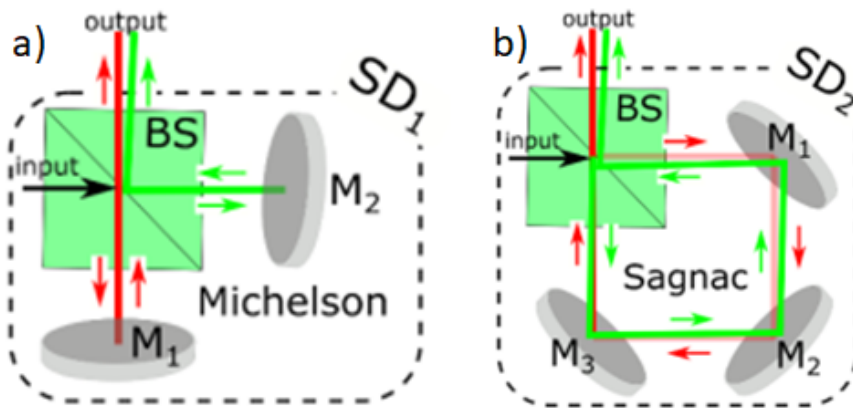


Figure 3.3. Comparison between two SD architectures for common path setups. BS - Beam Splitter. M1,2,3, Mirrors. (a) Michelson-based SD, allowing a quasi-common path configuration due to the slight separation of the optical paths between M1-2 and the BS. (b) Sagnac-based SD allowing full common path setups with completely overlapping optical paths for the object and reference waves.

optical paths consisting of identical components, respectively the clockwise path $BS \rightarrow M_1 \rightarrow M_2 \rightarrow M_3 \rightarrow BS$ and the counterclockwise path $BS \rightarrow M_3 \rightarrow M_2 \rightarrow M_1 \rightarrow BS$. The lateral displacement needed for the off-axis geometry is provided slightly tilting the [BSP](#). The involvement of total-common path architectures allows to use less coherent light sources such as led diodes, lowering the price of the setup and in principle raising the resolution. Comparing the performances of the presented setup and of the Mach-Zehnder introduced in Chapter 2, a sensible loss in the performance can be noted, as illustrated in Fig. 3.4 depicting two holograms acquired with the two setups (right, Mach-Zehnder, left, quasi-common path). The resolution of the setup is lowered due to the 2.6 times smaller

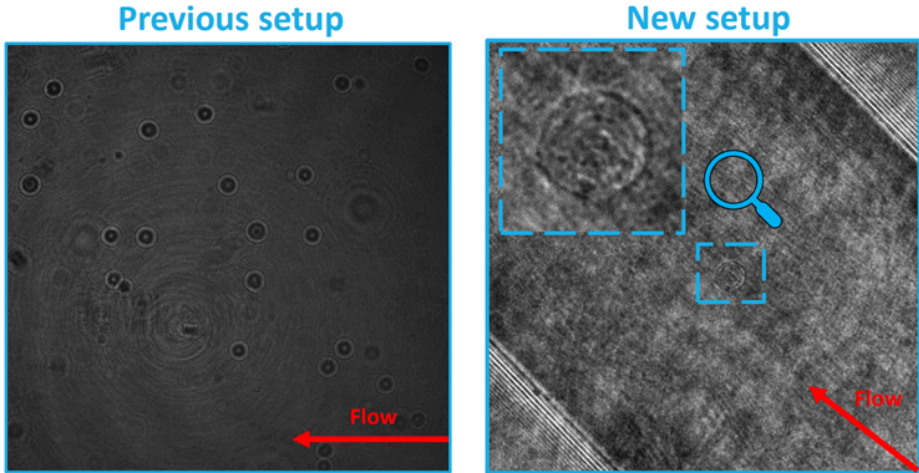


Figure 3.4. A comparison between the digital holograms acquired with the Mach-Zehnder setup (left) and with the common path setup (right) available at ISASI-CNR, with a detailing over the flow direction.

[NA](#), leading to worse quality holograms; the [FOV](#) of the camera is reduced of a 2.5 factor, leading to fewer projections acquired. This loss in the optical performance must be compensated by the numerical processing which must be more powerful, in accordance with the paradigm of Computational Tomography. Concerning the preparation of the data, detailed information can be found in [225]. The digital holograms, acquired via the setup sketched in Fig. 3.2 presenting the 45-degree tilted configuration,

are processed according to the numerical pipeline presented in chapter 2, which was experimentally tuned to adapt to the worse quality of the available holograms. In particular a bigger denoising parameter σ , accounting for the strength of the denoising effect, is used for the noise suppression stage based on the 2D Windowed Fourier transform and above described, experimentally chosen as $\sigma = 3$. At the end of the processing pipeline, the retrieved QPMs are back-rotated in order to align the flow direction to the y axis sketched in Fig. 3.5, left. However, the rolling angle recovery procedure has to be adapted to the tilted configuration as the tracking of the cells in this case is made in the 45-degree tilted coordinate system.

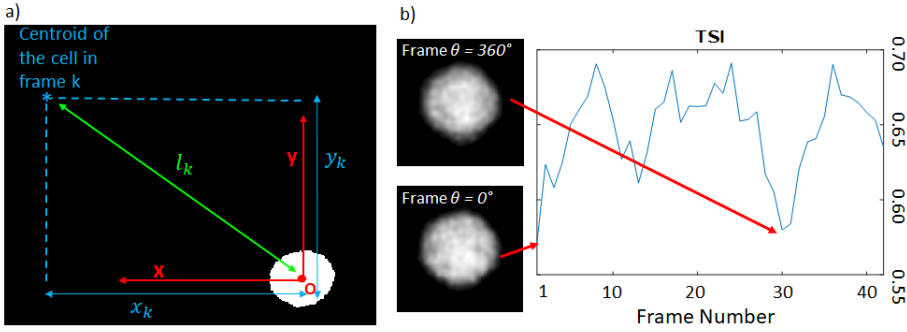


Figure 3.5. Rolling angle recovery procedure for the 45-degree tilted flow direction. (a) Coordinates used for the identification of the 360 degrees QPM. (b) TSI profile, where the frames assigned to the 0 and 360 degrees QPMs are highlighted.

3.1.2 Rolling angle recovery in 45 degree tilted system

As previously discussed, the first step for the rolling angle retrieval consists in the identification of the frame at which the image of the cell is rotated of 360 degrees in respect to the first frame, taken as the reference image. We term this frame f_{360} . The retrieval of f_{360} is made according to the Tamura Similarity Index (TSI) metric [211]. Given the reduced resolution of the holograms, the profile of the TSI shows a noisier profile if compared to the average ones arising from the Mach-Zehnder setup, resulting in a potentially lower precision in detecting the frame in which

the sample rotation occurred. However, from an experimental campaign it resulted that the **TSI** metric curve always exhibits a global minimum which allows to assign f_{360} without ambiguity. It is important noticing that since there is no fine control over the rotation velocity of the cells into the microfluidic channel, the full 360 event might be missed. Accordingly, the numerical reconstruction approach for the tomogram must be able to account for these errors. This scenario was already investigated in [211], in which it was demonstrated that, in similar microfluidic conditions and cell velocity ranges, the possible error in estimating the rotation angles provided negligible distortions in the reconstructed tomograms. For each recorder hologram, the cell is tracked as it moves into the **FOV** by following the coordinates of its centroid. Assuming the proportionality between the rotation and the translation of the cell the entire angular sequence can be reconstructed in the tilted geometry according to 3.1

$$\begin{cases} \theta_k = 0^\circ & k = 1 \\ \theta_k = 360^\circ \frac{l_k}{L_{360}} & k > 1 \end{cases} \quad (3.1)$$

In Eq. 3.1, θ_k is the rolling angle corresponding to the k frame and l_k is the distance between the centroids of the cell between the first available frame ($k = 1$) and the frame k . l_k is evaluated as $l_k = \sqrt{x_k^2 + y_k^2}$, where x_k and y_k are the coordinates of the centroid of the k^{th} frame in the introduced coordinate system (Fig. 3.5) and L_{360} corresponds to l_k evaluated for f_{360} . For the considered setup, given the reduced **FOV** of the camera, the average angular steps between frames is 6° .

3.2 Tomographic reconstruction via high order TV

The **FBP** algorithm described in Chapter 2 is the gold standard reconstruction technique for tomography. However, this algorithm notoriously is unable to produce high quality reconstructions when few, noisy projections are available [211]. Those, however, are the conditions we deal with for tomographic data acquired with the presented common path setup in which, as discussed, few projections are available due to the reduced **FOV** and the acquired data suffer from the reduced resolution of the entire setup. Therefore, more powerful approaches must be considered for the reconstruction

of the tomogram incorporating regularisation terms. As introduced in the previous Chapter, a common choice is to incorporate a TV regularisation term into the reconstruction. Even though this regularisation allows to obtain solutions with sharp edges even in the case of limited number of orientations, the underlying hypothesis it supposes is that the searched solution is piecewise constant function with a sparse gradient. However, the majority of the biological specimens do not fall into this category, as they show a smoother internal RI profile. As a consequence, the solutions provided by TV applied to biological tomographic reconstruction presents the typical cartoon-like, blocky artifacts that corrupt the reconstruction. This effect, generally called staircase effect, derives from the characteristic of the TV solver to approximate smooth regions and ramps as stairs, which are the piecewise constant regions supposed by the algorithm. Therefore, in the literature TV is mainly used as post processing step for restoring the quality of the reconstructions obtained with other solvers, instead as an inversion technique itself [116]. Numerous approaches have been proposed in the literature to deal with this problem. Among others, total generalised variation, which generates a piecewise (typically quadratic) polynomial approximation in smooth regions, was developed in [226] and the wavelet domain was also considered as an interesting sparsity-inducing transform [227]. However, one class of particularly interesting solutions is the family of High Order (HO) methods [226, 228, 229]. HO methods propose to overcome the staircasing effect introduced by TV introducing regularisation terms that tackle also derivatives of greater orders, in addition to the first order ones. Consequently, the space of the searched solution is not limited to the bounded variation space, allowing smoother RI profiles to be reconstructed. Although many HO approaches have been proposed in the literature, here we refer to a particular one which was developed by [230, 231, 232, 233, 234] called Polynomial Annihilation (PA). PA offers the advantage of adapting well to cases of under-samples and noisy Fourier data. Tomographic data, indeed, exhibit a Fourier-described link between the acquired measurements and the smooth-varying RI profile to reconstruct, as highlighted by FST and FDT. In addition, in the case of data arising from the presented common path setup the measured data are undersampled and noisy. In this work, for the first time in TPIFC, an HOTV approach was used for the reconstruction of tomograms.

3.2.1 High Order Total Variation via Polynomial Annihilation transform

PA was first proposed for 1D signals [233]. Later on it was extended in [234] to multi-dimensional spaces such as images by incorporating a particularly efficient numerical implementation based on the Split Bregman algorithm, which was introduced in [235]. PA regularisation encourages solutions with sparse boundary regions, as happened for TV. However, while TV regularisation is designed under the assumption that away from these boundaries the solution is essentially a piecewise constant function, i.e. a polynomial of degree zero, PA assumes that the searched solution is a piecewise polynomial of greater degree, which allows a more accurate reconstruction for a wider class of images. Sanders in work [230] applied for the first time the PA transform to the reconstruction of under resolved electron tomography data. Moreover, in [236], he realised a numerical implementation of the PA transform based on the implementation of the TV made by [137] and discussed in Chapter 2. He named the software High Order Total Variation and realised it as an open source code. In this work, for the numerical reconstruction of our data we will refer to this numerical implementation in [236], version 2. Hence, we briefly outline the author's description of the PA transform. The generic tomographic reconstruction problem seeks to recover $f \in R^{n \times n}$, where $f_{\{i,j\}}$ represents the image intensity at pixel (i, j) . If needed, f can be also represented as a vector $f = \{f_i\}_{i=1}^{n^2}$. As mentioned in Chapter 2, given the ill-posedness nature of the problem, it is generally solved embedding a regularisation term in the solution as in Eq. 3.2,

$$J(f) = \|Af - g\|_2^2 + \mu T(f) \quad (3.2)$$

where A represents the measurements matrix, g the available measurements, μ is the regularisation parameter and $T(f)$ a suitable regularisation term. In the case of the PA transform, the regularisation term is defined as

$$(T_k f)_j = \sum_{m=0}^k (-1)^{k+m} \binom{k}{m} f_{j+m} \quad (3.3)$$

for indices $j > N - k$, $T_k f$ can be defined with a periodic extension of f . Loosely speaking, T annihilates f at grid points where locally f is a polynomial less than a desired degree which is called order of the PA operator k . For $k = 1$, PA reduced to the standard TV. As with TV, the PA regularisation encourages sparse boundary regions. However, in contrast with TV, the smooth regions are modelled as polynomials rather than constant functions. The annihilation effect is due to the equivalence of the PA transform in Eq. 3.3 with a finite difference operator. Indeed, considering a function f defined at equally spaced grid points, by defining the finite difference operator

$$\begin{cases} D_h^1 f(x) = D_h f(x) = f(x+h) - f(x) \\ D_h^{k+1} f(x) = D_h^1(D_h^k f(x)), k \geq 1 \end{cases} \quad (3.4)$$

If f is a polynomial of degree less than k in the interval $[x, x+kh]$, the finite difference operator is equal to 0. It can be demonstrated using induction that Eq. 3.4 can be written as

$$D_h^k f(x) = \sum_{m=0}^k (-1)^{m+k} \binom{k}{m} f_{x+mh} \quad (3.5)$$

Therefore, the PA operator coincides with the finite difference one which defines a sparsifying transform. For multiple dimensional higher order finite difference, T_k is extended along each dimension as $T_k = [T_k^x, T_k^y]$, where T_k^x, T_k^y compute the finite difference operator along the x and y dimension respectively.

3.2.2 Numerical assessment of HOTV

To evaluate the effectiveness of the chosen regularisation method and fine-tune the parameters for HOTV reconstruction, the numerically simulated cell model discussed in Chapter 5 is employed, which simulates realistic tomograms as they would appear after undergoing a real tomographic reconstruction process in a real setup. The simulated RI distribution is in the range $[1.334, 1.410]$. Fig. 3.6 provides a visual representation of the central slice and an isolevel visualisation of a sample cell generated with our model, serving as the ground truth RI distribution. For the

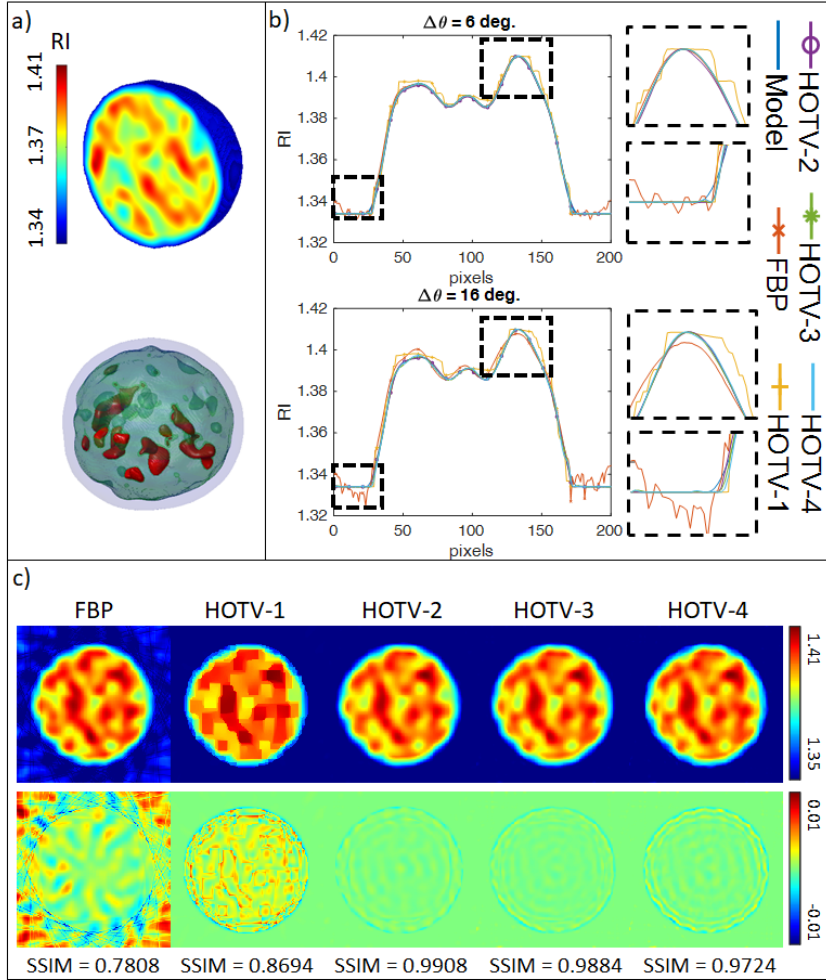


Figure 3.6. Numerical simulation for the assesment of the **HOTV** performance. (a) 3D (top) and isolevel (bottom) visualisation of the simulated cell. (b) Horizontal cut through the central slice of the reconstructed tomograms for $\Delta\theta = 6^\circ$ and $\Delta\theta = 16^\circ$ considering different solvers. (c) Top row: central slice of the reconstructed tomograms considering different solvers for $\Delta\theta = 16^\circ$, bottom row: difference slice between the reconstructions obtained with different solvers and the simulated model. Length of the reference scale bar is $5 \mu\text{m}$. Image reproduced from reference [225].

isolevel visualisation, two internal thresholds set at 0.33 and 0.66 relative to the maximum simulated **RI** value were applied. The performance of two reconstruction methods was compared: **FBP**, which is the ground truth reconstruction technique, and **HOTV**. The **HOTV** method was tested at different orders, including **HOTV1** (equivalent to the conventional **TV** approach), **HOTV2**, **HOTV3**, and **HOTV4**. The **HOTV** reconstruction process involved various parameters, including data fidelity term μ for balancing data and regularisation terms, the **HOTV** order k , the number of iterations for inner and outer loops, and termination tolerances. The default settings for all parameters was retained except the number of inner and outer iterations, both set to 30, and the regularisation parameter μ was adjusted to optimise the tomographic reconstruction's fidelity compared to the ground truth: $\mu = 24$ for **HOTV1**, $\mu = 48$ for **HOTV2**, $\mu = 96$ for **HOTV3**, and $\mu = 192$ for **HOTV4**. Furthermore, all reconstructions adhered to a non-negativity constraint, and only the projection angles ranging from 0 to 360 degrees were included in the reconstruction. Simulations considered different angular step sizes, specifically $\Delta\theta = 6^\circ$ (referred to as case A in Fig. 3.6 (b)) and $\Delta\theta = 16^\circ$ (referred to as case B in Fig. 3.6 (c)), corresponding to the average and maximum angular steps observed in realistic sequences for the considered setup. In case A, the tomogram reconstruction was made with 60 projections, while in case B, only 22 projections were available. In both of the scenarios examined, **FBP** exhibited noticeable noise issues, particularly in the outer regions of the reconstructed tomogram, as depicted in the lower insets of Fig. 3.6 (b), (c). In contrast, the **HOTV** solvers effectively smoothed out the noise in these external regions. Furthermore, in case B, where there was a reduced amount of available data, **FBP** struggled to accurately recover the **RI** structures of the simulated cell. This deficiency is evident in the bottom row of Fig. 3.6 (c). As anticipated, even **HOTV1** yielded unsatisfactory results since it tends to produce a piecewise constant solution, which is not well-suited for describing the intricate anatomy of typical cells. On the contrary, **HOTV2**, **HOTV3**, and **HOTV4** solvers performed admirably. To assess the performance of the different solvers, the **SSIM** was employed to measure the similarity between the ground truth and the reconstructed tomograms. The results were as follows: **FBP** achieved an **SSIM** of 0.7808, **HOTV1** scored 0.8694, **HOTV2** achieved a high **SSIM** of 0.9908, **HOTV3**

reached 0.9884, and [HOTV4](#) obtained a score of 0.9724. These findings unequivocally demonstrate that both [HOTV2](#) and [HOTV3](#) outperformed both [HOTV1](#) and [FBP](#) in terms of accuracy, with [HOTV2](#) showing slightly superior results compared to [HOTV3](#). While [HOTV4](#) yielded slightly less accurate results due to some artefacts resulting from the broader variability of the sought after parameters, [HOTV2](#) emerged as the clear choice with the best performance among the numerically evaluated [HOTV](#) algorithms. To further investigate the performance of [HOTV2](#), a numerical assessment was performed concerning the robustness of the algorithm towards mistakes in the estimation of the 360-rolling angle frame. In particular, two cases were modelled: in the first one, an angular step of 7° was considered, in the second one, an angular step of 11° was considered, to build two angular sequences termed "sequence7" and "sequence11". In both cases the 360 frame is not sampled. The [QPMs](#) are simulated using "sequence7" and "sequence11". Differently from the analysis reported in [211], in our case, given the elevate angular step, the error in the estimation of the 360 frame is in the worst case committed assigning it to the frame before or after the real one. Accordingly, for each case two simulations were performed, assigning the 360 orientation to the closest angle before 360 (354° for "sequence7" and 352° for "sequence11") and the closest angle after 360 (364° for "sequence7" and 363° for "sequence11"). The retrieved reconstruction angular sequences, termed "sequence7Rec" and "sequence11Rec" are built as described sec. 3.1.2. Finally, the [HOTV2](#) tomograms were reconstructed considering the two angular sequences "sequence7Rec" and "sequence11Rec". As a reference, the [HOTV2](#) tomograms obtained using the correct angular sequences, "sequence7" and "sequence11", were used to evaluate the rms% normalised corresponding to the wrong reconstruction sequence, whose values are reported in table 3.1. It is observed that the greater errors are committed when the 360 frame is over estimated.

Angular step	Estimated 360 frame	rms%
7°	357°	3.06
7°	364°	5.22
11°	352°	2.84
11°	363°	6.02

Table 3.1. Numerical assessment of the performance of **HOTV2** in case of a wrong estimation of the 360 frame. The rms% normalised computed between the reconstructions obtained using the corrected angular sequence and the wrongly reconstructed one is presented.

Finally, Fig. 3.7 reports a plot of the rms% vs the mean value of **RI** of the slice of the tomogram. The rms% was computed only for the internal area of the cell, selecting it through a binary mask, and only for the slices 0 to 100 given the almost symmetrical behaviour of the mean **RI**. As suggested by the curves, the error is more elevate in the most peripheral slices and lowers progressing towards the central ones. The plot also highlights the difference in terms of rms between **FBP** and **HOTV2**.

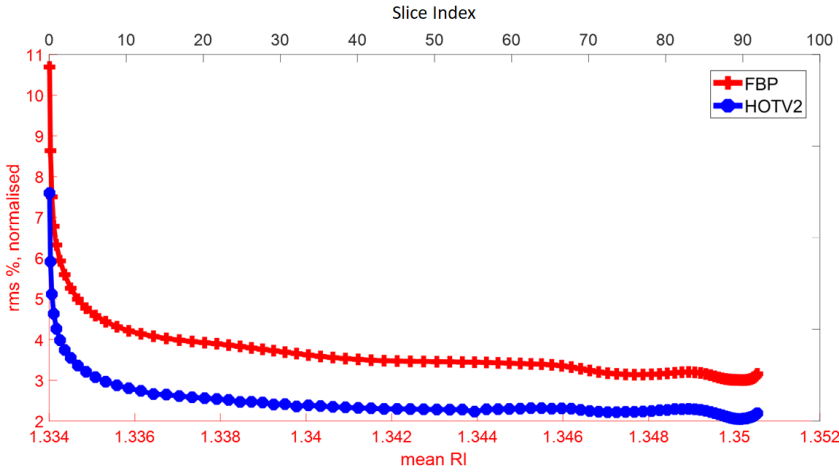


Figure 3.7. Plot of rms%, normalised vs mean value of **RI** evaluated for different slices of the tomograms reconstructed via **FBP** and **HOTV2**.

3.2.3 Experimental validation of the HOTV

The obtained **QPMs** cropped to a square area of 200×200 pixels around the cell centroid equivalent to $20 \times 20 \mu m$ and the retrieved angular sequence were used as the input parameters of both the gold-standard **FBP** and **HOTV2** tomographic reconstruction algorithms, respectively. Only the available projections between 0 and 360 degrees were used as for the simulate case. Fig. 3.8 presents the reconstructions obtained using the two

algorithms. First, the tomograms of monocytes observed rolling in the microfluidic channel were reconstructed. Cross-section slices of four of the tomograms generated using **HOTV2** are shown in Fig. 3.8(a1–d1), while those reconstructed using **FBP** are displayed in Fig. 3.8(a2–d2). **HOTV2**

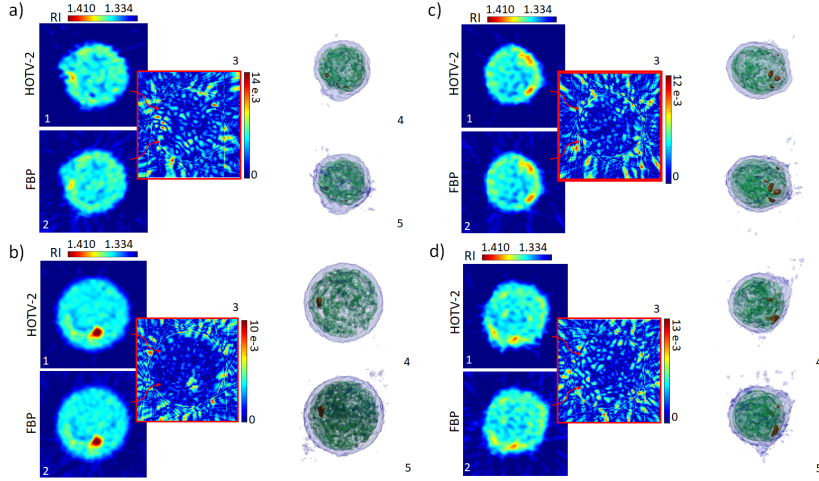


Figure 3.8. Tomograms of four different monocytes reconstructed. Each panel (a–d) shows cross-section slices from the **HOTV2** tomographic reconstruction (subfigures (a1)–(d1)), cross-section slices from the **FBP** tomographic reconstruction (subfigures (a2)–(d2)), and the absolute value of the difference between the two cross-section slices (subfigures (a3)–(d3)), together with isolevel visualizations for the **HOTV2** (subfigures (a4)–(d4)) and **FBP** reconstructions (subfigures (a5)–(d5)). The length of the reference scale bar is 5 μm . Fig. reproduced from reference [225].

reconstructions exhibit a homogeneous background, contrasting with the **FBP** results, which were discussed earlier in our numerical simulations. Additionally, **HOTV2** appears to offer higher **RI** contrast compared to **FBP**, which produces smoother **RI** profiles. The beneficial effect of **HOTV2** over **FBP** is evident when examining the absolute difference between the two cross-section slices (Fig. 3.8(a3–d3)), termed absolute error, highlighting **HOTV2**’s ability to reduce artefacts introduced by **FBP** both in the internal region of the cell and the background. Insights into cell anatomy are visualised in the isolevel plots for both algorithms, **HOTV2** (Fig. 3.8(a4–d4))

and FBP (Fig. 3.8(a5–d5)). Fig. 3.9 provides histograms of conventional morphological features, such as average RI (Fig. 3.9 (a), biovolume Fig. 3.9 (b), equivalent diameter Fig. 3.9 (c), and dry mass Fig. 3.9 (d)), evaluated across all examined monocytes in the case of HOTV2 reconstructions. These features are crucial for label-free single-cell analysis, encoding infor-

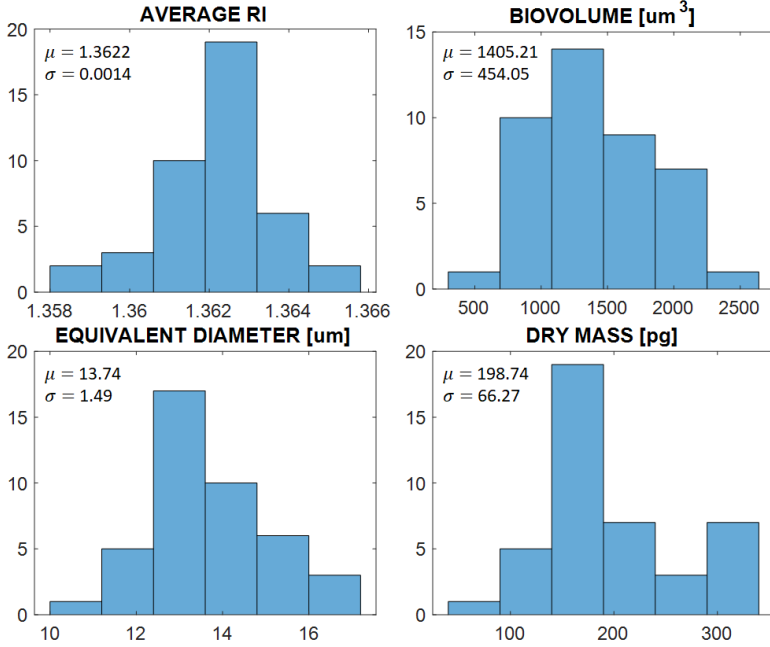


Figure 3.9. Histograms computed over the population of monocytes under examination; (a) Average RI. (b) Biovolume. (c) Equivalent diameter. (d) Dry mass. Fig. reproduced from reference [225].

mation about cell cycles and playing a pivotal role in cell phenotyping and disease identification. The mean value μ and standard deviation σ of these histograms align with expected values reported in previous studies. To further validate our approach, a similar analysis was conducted on a different cell line, mouse embryonic fibroblasts NIH-3T3, as shown in Fig. 3.10. The results, depicted in Fig. 3.10 (a) and (b), highlight the advantages of

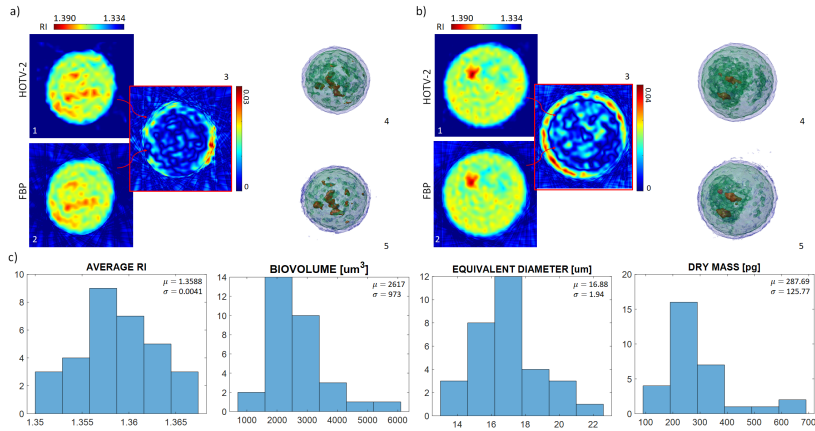


Figure 3.10. Tomograms of two NIH-3T3 cells. For both panels (a,b), cross-section slices from the **HOTV2** tomographic reconstruction (subfigures (a1),(b1)), cross-section slices from the **FBP** tomographic reconstruction (subfigures (a2),(b2)), and the absolute value of the difference between the two cross-section slices (subfigures (a3),(b3)) are presented, together with isolevel visualisations for the **HOTV2** (subfigures (a4),(b4)) and **FBP** reconstructions (subfigures (a5),(b5)). (c) Histograms of average refractive index, biovolume, equivalent diameter, and dry mass, computed over the population of NIH-3T3 under examination. Image reproduced from reference [225].

HOTV2 over FBP, consistent with our earlier observations. Fig. 3.10 (c) provides histograms for the same morphological parameters, now calculated across the entire population of examined NIH-3T3 cells, with values falling within the expected range according to the literature. Figs. 3.9 and 3.10 showcase morphological features that can be used to differentiate between the two analysed cell lines. Fig. 3.11 (a–f) displays scatterplots among these features, revealing strong correlation between biovolume and equivalent diameter, as indicated by their quasi-linear trend in Fig. 3.11 (f). This suggests that the pair of scatterplots in Fig. 3.11 (b),(d) and Fig. 3.11 (c),(e) represent similar population distributions. Notably, NIH-3T3 cells exhibit a wider morphological variability compared to monocytes, indicating greater heterogeneity within the NIH-3T3 population. In Fig. 3.11 (g) and 3.11 (h), the possibility of data clustering was explored using Principal Components Analysis (PCA) and t-Distributed Stochastic Neighbour Embedding (T-SNE) visualisation. While clustering appears feasible with these morphological features alone, a more accurate classification of different cells would require deeper tomographic data analysis, including advanced 3D image features and/or suitable artificial neural networks.

3.2.4 Repeatability of the measurements

In our experimental setup, it was possible to image the same cell multiple times. The microfluidic flow cell unit used in our setup allows for precise control of pressure within the microfluidic chip, which, in turn, regulates the speed of cells in the flow. By adjusting the flow unit apparatus appropriately, the direction in which cells rolled was reversed, achieving both forward and backward flow directions by changing the pump pressure. This allowed us to move a cell back to its initial position and observe it repeatedly while maintaining the same flow direction. Consequently, rolling angles for five different observations of the same monocyte cell were calculated, leading to five independent tomographic reconstructions (Fig. 3.12 (a) and 3.12 (b)). Cross-sections through these tomograms, generated using HOTV2, are presented in Fig. 3.12 (a). In the top row of Fig. 3.12 (a), you can see the central x-z slice for each occurrence, revealing the same internal structure across observations. The bottom row shows a different x-z slice, highlighting a distinctive feature of the cell's external shape. It's

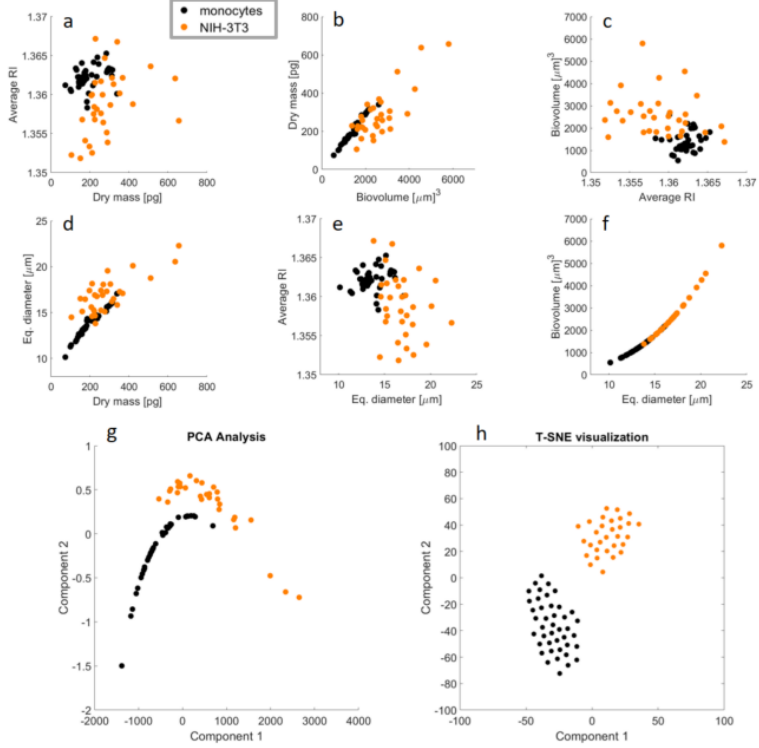


Figure 3.11. Data analysis comparing monocytes and NIH-3T3 cell lines; (a-f), scatterplots between each pair of the conventional morphology features calculated for the analyzed cell lines, i.e., average RI, Dry Mass, biovolume and equivalent diameter. In particular, subfigure (f) shows a quasi-linear trend, due to the obvious correlation between biovolume and equivalent diameter. This renders redundant the information content provided by the pair of subfigures (b) and (d) as well as (c) and (e), which display similar population distributions. Subfigures (g, h) show two of the most popular methods for inspecting high-dimensional data, i.e. PCA analysis and the T-SNE visualisation, respectively; these have the effect of removing such redundancy and show the spatial separation of the two analysed cell populations, thus demonstrating the possibility of clustering them. Fig. reproduced from reference [225].

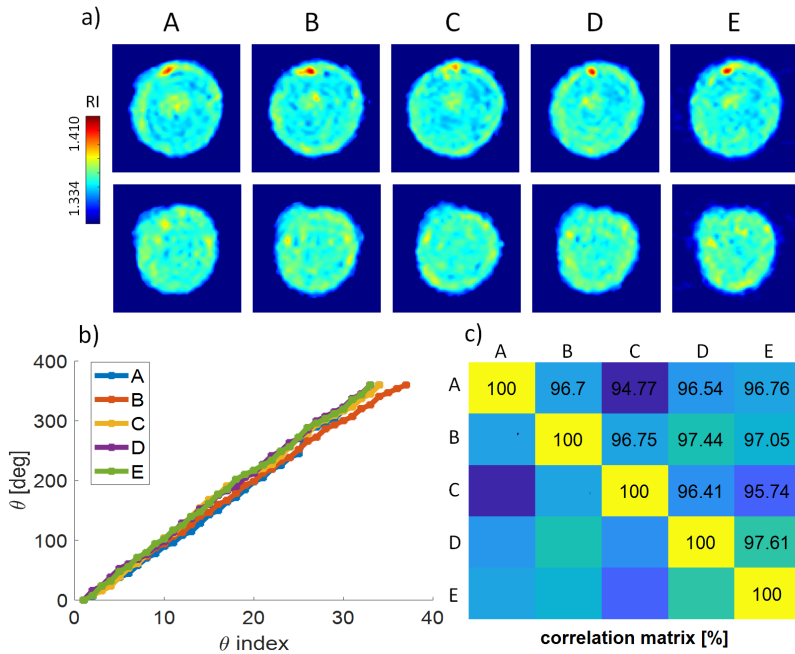


Figure 3.12. (a–c) Repeatability of the experimental reconstruction process. (a) Comparison between reconstructions of the same tomograms for five different observations of the same cell [A–E] which is rotated five times through the channel: central (top row) and peripheral (bottom row) x-z slices of the tomograms obtained by HOTV2. (b) Retrieved angular sequences for the five considered experiments. (c) Correlation matrix calculated between the tomograms reconstructed for the five considered independent observations. Image reproduced from reference [225].

important to note that some variation among individual observations may occur due to factors affecting cell anatomy, such as cell deformation caused by hydrodynamic forces or adhesion to the channel [237]. Nonetheless, the repeatability of the measurements was assessed by using the correlation coefficient as a similarity measure among all five individual tomograms obtained for the same cell displayed in Fig. 3.12. To do that, the 3D tomograms were first realigned to obtain matching visualisations. This step is necessary as the five different experimental measurements lead to shifted angular sequences reconstructed due to the assumption of the first available frame as the $\theta = 0^\circ$ frame, resulting in rotated tomograms. The average value of correlation coefficient evaluated for the five realigned 3D tomograms of the same cell is 96.6%. The correlation matrix (Fig. 3.12 (c)) illustrates a strong agreement, with all values exceeding approximately 95%, indicating the robustness of our experimental recordings and numerical processing. Generally, the values of correlation coefficient computed between different cells of the same line is around 70%. This provides a reference value for the calculated correlation coefficient, demonstrating the consistency of our experimental and analytical procedures.

3.2.5 Use of fractional calculus in the HOTV reconstruction

Recently, solutions exploiting fractional derivatives have been proposed for computer vision applications [238, 239, 240, 241, 242, 239, 243], as the expectation is that the use of this theory will lead to a much more elegant and effective way of treating the staircase effect. Specially, the fractional-order TV models play an important role for image denoising, inpainting, and motion estimation [244, 245, 246, 247, 248] and many approaches have been introduced in the literature concerning this topic [249]. The possibility to evaluate the PA term for fractional orders of derivatives allows the interesting option to incorporate fractional calculus into the HOTV framework too. Fractional calculus is an ancient branch of mathematics that studies the possibility of defining real or complex powers α of the differentiation and integration operators. The most interesting property fractional derivatives exhibit is that they are local operators only for integer values of α , as the definition coincides with the limit of the incremental ratio as for the standard derivative, whereas for non-integer values

of α the values of the fractional derivative of a function f in a point x also depend on points far from x . Many mathematical definitions of the fractional derivatives exists [250], however, here the approach discussed in [251] is followed, where the authors efficiently incorporated fractional calculus into the TV minimisation. The modelling of the problem follows the same scheme previously introduced for TV incorporating the fractional order derivative term. According to [251], in this case we seek the minimization of Eq. 3.6

$$\tilde{f} = \arg \min_{u \in \Omega} \{E(u) := TV^\alpha(f) + \frac{\mu}{2} \|Af - g\|_2^2\} \quad (3.6)$$

In which TV^α is the fractional order TV operator defined as in Eq. 3.7

$$TV^\alpha(f) = \|\sqrt{(D_1^\alpha f)^2 + (D_2^\alpha f)^2}\|_1 \quad (3.7)$$

In Eq. 3.7 $\sqrt{(D_1^\alpha f)^2 + (D_2^\alpha f)^2}$ is the discretisation of the $|D^\alpha f|$, D^α is the fractional order derivative operator and $D^\alpha f := (D_1^\alpha f, D_2^\alpha f)^T$. D_1^α and D_2^α are linear operators corresponding to horizontal and vertical fractional-order derivative. Using the Grunwald-Letnikov definition of fractional derivatives [252, 247], the finite fractional-order forward difference can be obtained by Eq. 3.8:

$$\begin{cases} D_1^\alpha f_{i,j} = \sum_{k=0}^{K-1} w_k^{(\alpha)} f_{i+k,j} \\ D_2^\alpha f_{i,j} = \sum_{k=0}^{K-1} w_k^{(\alpha)} f_{i,j+k} \end{cases} \quad (3.8)$$

Having defined $w_k^{(\alpha)} = (-1)^{k+1} C_k^\alpha$, $C_k^\alpha = \frac{\Gamma(\alpha+1)}{\Gamma(k+1)\Gamma(\alpha-k+1)}$ as the generalized binomial coefficient and Γ as the Gamma function. The $w_k^{(\alpha)}$ can be also obtained recursively as $w_0^{(\alpha)} = -1$, $w_k^{(\alpha)} = (1 - \frac{\alpha+1}{k})w_{k-1}^{(\alpha)}$, $k = 1, 2, \dots$. This definition coincides with the standard definition of derivative for an integer order α . As proceeded in the previous Sections, tomograms of rolling WBCs were reconstructed exploiting HOTV with fractional order derivatives. Indeed, given the proximity of the values of SSIM computed in the range [2, 3] for the HOTV reconstruction for different orders reported in Fig. 3.6, a deeper investigation is conducted in that interval. Fig. 3.13 shows the results of the investigation, where the quality of the tomographic reconstructions using fractional orders in the mentioned range is assessed.

The plot in Fig. 3.13, subfigure (a) shows the SSIM values computed for the reconstructions obtained using fractional orders and the ground truth. From the SSIM values, it can be established that the best order of total variation to exploit is the order 2.3. All the orders allow to obtain high quality reconstruction, as illustrated in Fig. 3.13, subfigures (b) where an horizontal cut taken from the central yz slice of the reconstructions is shown, but the choice of the intermediate order is optimal as shown in the enlarged box in Fig. 3.13, subfigure (c). Fig. 3.13, subfigure (d) shows the central yz slice of the reconstruction obtained for the integer HOTV orders 2 and 3 and the fractional order 2.3. Subfigure (f) shows the ground truth corresponding slice. Considering the difference slice between the ground truth and the reconstruction obtained for HOTV2, HOTV2.3 and HOTV3 and shown in Fig. 3.13 (e), the beneficial effect of the 2.3 order in terms of internal reconstruction fidelity is appreciated, as confirmed by the smoother profile of the difference slice.

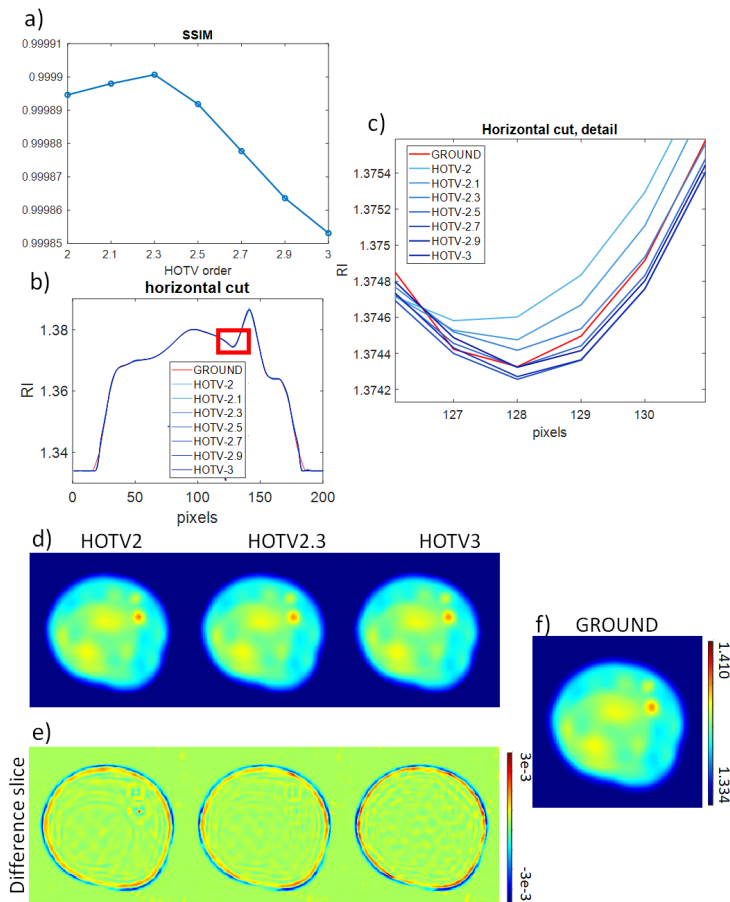


Figure 3.13. Comparison between fractional orders of **HOTV** tomographic reconstructions. (a) SSIM compared between the ground truth simulated 3D tomogram and the **HOTV** reconstructions for different fractional orders. (b) Horizontal cut for a yz tomographic reconstruction slice. (c) Expanded detail in the red box in subfigure (b), showing the different performance of the various orders. (d) Central yz slices of the reconstruction obtained using **HOTV2**, **HOTV2.3** and **HOTV3**. (e) Difference slice between the considered reconstructions and the ground truth image. (f) Central yz slice of the ground truth simulated cell.

Chapter 4

AI-powered DHM and TPIFC

The advances brought by [AI](#) in the field of image processing have revolutionised the fields of biomedical optics [\[253\]](#) and medical investigation. One of the most promising applications is tomography and [CNNs](#) have been successfully integrated in the reconstruction framework at different stages. Concerning the field of [ODT](#), a vast literature exists, in particular relative to the problem of missing cone. [DL](#)-based approaches have been used for the first time to retrieve the 3D [RI](#) distribution in [\[254\]](#), where starting from 2D strongly undersampled measurements the authors could retrieve the 3D tomograms outperforming the Rytov approximation-based solvers. The training was performed using simulate data, with the advantage of being able to build an ad hoc dataset, but proved optimal generalisation capabilities over experimental data too. This first demonstration paved the way to the development of similar methods for the inversion of [ODT](#) data [\[255\]](#). Other groups proposed to use [DL](#) methods to enhance the quality of the output obtained using conventional solvers, such as in [\[256\]](#) where a 3D [CNN](#) is trained to replicate the [TV](#) reconstructions or to accelerate specific bottlenecks of the [ODT](#) reconstruction framework [\[257\]](#). On the other hand, while a reduced literature is present for [TPM](#), many solutions can come from the field of medical imaging and in particular [CT](#) and Magnetic Resonance Imaging ([MRI](#)) reconstruction [\[258\]](#). Indeed, in this field [DL](#) has been intensively exploited to optimise the inversion process or to enhance the quality of the reconstruction for cases of low-noise or [LAT](#) [\[259\]](#). Moreover, also the problem of lost resolution can be faced through

DL. Super resolution techniques have been successfully incorporated into biomedical imaging to numerically recover the resolution which might be lost due to a less-performing acquisition procedure. This problem also affects **TPIFC** in experimental realisations such as the common path-based one at ISASI-CNR introduced in Chapter 3, where due to the choice of optical components the resolution is reduced. Object of this Chapter will be the application of a technique developed in the framework of **CT** to the Radon inversion problem in **TPIFC**, optimised focusing on a lightweight tool suitable for **LOC** applications. In addition, the problem of super resolution in **TPIFC** will be addressed as a preliminary analysis, presenting some early results.

A different but particularly interesting application of **AI** regards the field of single cell analysis [260, 261] and its use in automated diagnosis [262]. In particular, the diagnostic technique **LB** is strongly benefiting of the integration of **AI**-aided methods. **LB** recently arose as a powerful alternative to tissue biopsy as a support in the identification and diagnosis of many diseases [209]. The strength of **LB** is that it greatly suits **IFC**, the modern state-of-the-art cell analysis tool. In fact, the amount of data provided by **IFC** in a short amount of time allows to successfully couple it with **AI** approaches which can be integrated into diagnostic, from detection of cells to discrimination and diagnosis support. The possibility to conjugate **AI** methods with label free, **QPI** techniques is particularly interesting as the information contained in **QPMs** might positively affect the results. As accurate cell identification and characterisation is critical for understanding the psychophysiology of various diseases, including cancer, in this elaborate this aspect is investigated, exploiting fully the information contained into the **QPMs**. Two problems of cell population classification are in fact discussed. In the first case, an exploratory analysis is conducted to analyse the informativeness brought by **QPI** in an application aiming to identify with the lowest number of false negatives **CTCs** of ovarian cancer cell. Instead, in the second application the informativeness of the 3D encoded information contained into **QPMs** is discussed as a lightweight alternative to tomography.

The Chapter is structured as follows. First, a reliable numerical model of a tomogram is developed, with the idea of obtaining a simulation as similar as possible to a numerical reconstruction of experimental data acquired

with the setups available at ISASI-CNR. The availability of a reliable simulate model is crucial when concerning DL approaches, as the training procedure is strongly facilitated by it and quantitative considerations can be made over the reconstruction tools.

Following, a DL based procedure for the inversion of 2D Radon data is presented for the case of few data available. The proposed CNN is particularly lightweight and characterised by a small inference time, achieving the aim to go towards real-time processing, LOC oriented solutions. In addition, the procedure is generalised to full 3D data. Then, the problem of resolution enhancement is discussed as a preliminary stage analysis. Starting from literature approaches introduced for CT scan, some solutions are explored to recover the lost resolution due to a less performing optical setup.

Concluding, the two cell discussed classification approaches are presented.

4.1 Speeding up the tomographic reconstruction processing

Nowadays, one of the biggest challenges of medical diagnostic and in particular of single cell analysis is the possibility of conjugating fast acquisitions (high throughput) with a quasi-real time visualisation (very reduced reconstruction time), performed onto commercial LOC devices characterised by lower hardware performances. Working in these conditions generates criticisms in the tomographic reconstruction, as the fast acquisition comports a reduced number of orientations available, whereas the reduced optical performance decreases the quality of the acquired data. As a consequence, the tomographic problem results strongly ill-posed and must be dealt with accordingly. The setup introduced in Chapter 3 successfully achieves the concept of going towards LOC applications, given the possibility to be used outside of optical labs and its reduced price. The problem of reduced number of orientations available was dealt with incorporating high-order methods for the reconstructions which successfully overcome the drawbacks brought by the FBP reconstruction. However, the discussed solution is not compatible with real time applications and reduced hardware resources, as the computational burden and the reconstructions time are increased of several orders of magnitude respect FBP

being the inversion performed iteratively. In addition, it does not tackle the problem of recovering the lost resolution of the acquired holograms. Interesting solutions for these issues can come out by incorporating DL techniques into the tomographic reconstruction problem. The use of DL for solving the sparse-view tomographic problem in a short time and the recovery of the lost resolution in the reconstruction will be object of discussion of the this Chapter.

4.2 Simulated tomographic cell phantom

When discussing data-driven approaches, a crucial point is the availability of a dataset capable of representing correctly the problem considered and of a suitable dimension. Indeed, the possibility to generalize over different data and therefore obtain a flexible reconstruction method is entirely dependent on using a correct dataset. In the case of microscopic imaging, the problem is even more accentuated. This comes from the lack of an universally recognised ground truth as the knowledge of biological samples is linked to the visualisation technique used and it is impossible to correctly establish the internal distribution of the RI distribution. This not only constitutes a problem when introducing new numerical reconstruction approaches, as it is difficult to establish a reference to compare performances, but also when using data driven methods for the creation of the dataset. Concerning the latter problem, generally in the case of microscopic imaging two approaches are used. The first one requires to work with a fully experimental dataset. This approach, even though apparently the most correct one, has intrinsic disadvantages that must be carefully considered. First of all, a numerical method is needed to reconstruct experimental data. Accordingly, the numerical errors and bias introduced by any type of solver would be incorporated into the images of the dataset and any AI approach would learn considering those imprecisions as part of the problem. In addition, the size of the dataset would be dependent on the availability of experimental data which is related to the scheduling of the experiments, availability and preparation of the samples and laboratory times. Hence, it is often difficult to obtain large enough datasets. A second approach would be to use a fully synthetic dataset. This would allow to completely overcome the problem of the dimension of the dataset,

which might be enlarged as needed when needed; in addition, the model can be customised at each use to highlight the specific aspect we wish to tackle (e.g., resolution enhancement or quality improvement of the reconstruction). However, the use of a numerical dataset is fully dependent on a simulation which represents correctly the biological specimen of interest. This is a non-trivial problem as often the biological information available over the sample in terms of RI distribution and internal organelles does not translate into how the sample appears after being probed with a realistic optical system. Summarising, to deal with this problem the following procedure is used. Starting from a realistic cellular model developed by [263], the simulation was generalised to obtain a numerical model tomogram which resembles as much as possible a realistic reconstruction that could be obtained of a sample acquired with the Mach-Zehnder setup available at ISASI-CNR presented in Chapter 2, established as the reference setup possessed. Accordingly, here first the developed cellular model is introduced, and then the numerical transformation used to create the realistic-looking simulate tomogram is illustrated.

4.2.1 Cellular model

The simulated model used for a cell was developed starting from the work [264], where a confocal microscope was used to analyse MCF7 cells and to extract their 3D morphological structure. A population of MCF7 cells was stained using three fluorescent dyes to measure the average value and standard deviation of 3D morphological parameters of the overall cell and of main internal structures such as nucleus and mitochondria. These measurements have been here exploited to simulate a 3D numerical cell phantom. It is worth mentioning, however, that measuring accurate RI values at sub-cellular level is still a deeply debated topic [265, 266] and realistic RIs cannot be replicated as they are not yet well known. Concerning the developed model, the chosen pixel size is $1 \text{ pix} = 0.12 \mu\text{m}$ and the simulated cell has an overall volume of $256^3 [\text{pix}^3]$. The cell is composed of four sub cellular structures: membrane, cytoplasm, nucleus, and several mitochondria. Membrane, nucleus, and mitochondria have been shaped as ellipsoids with randomly chosen semi-axes length. The external shape of the cell was simulated without considering a canonical geometrical structure but an irregular shape which does not present symmetry. In this way,

each simulated cell is characterised by a different shape, facilitating the generalisation to real data. Finally, the cytoplasm is obtained through a morphological erosion of the cell shape. The number of simulated mitochondria can be customised for each simulation. The values of **RI** of the single sub-cellular components have been randomly drawn from normal distributions characterised by mean value μ and standard variation σ which are set in accordance to the values of literature [263]. The choice of the Gaussian distribution is in accord to the demonstrated physical distribution of the **RI** values discussed in [263]. The simulated **RI** ranges from $n_{min} = 1.334$ to $n_{max} = 1.410$. Fig. 4.1 presents an isolevel visualisation of a model tomogram of a cell simulated according to the described process.

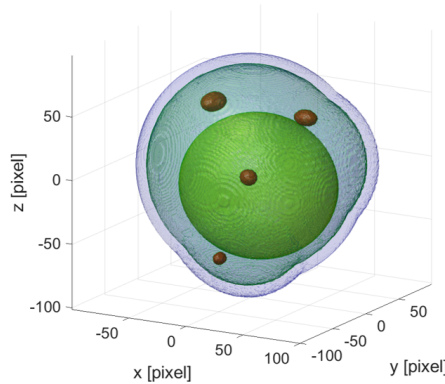


Figure 4.1. Example of a simulated model cell. The membrane, cytoplasm, nucleus and four mitochondria organelles are visible.

4.2.2 Realistic tomogram simulation

Once the realistic cell model is obtained with the described procedure, the simulation is numerically processed to emulate the effects of passage into a realistic optical system of the cells, in order to obtain simulations which are more resembling of real tomograms obtained with real acquisition setups. At this purpose, each internal zone of the modelled cell (membrane, cytoplasm, nucleus and mitochondria) is modified multiplying it by a numerical factor. This factor is generated creating a profile

with the same size of the internal zone in question and values drawn randomly from an opportune normal distribution. Each normal distribution used has mean value equal to the mean RI value of the internal zone of object and standard deviation experimentally tuned and set equal to $\sigma_{membrane} = \sigma_{cytoplasm} = \sigma_{nucleus} = 0.5, \sigma_{mitochondria} = 0.2$. The generated random factor is then downsampled and upsampled of an opportune quantity, experimentally established as 20. This step is used to simulate the effects of the PSF of the setup. As a last stage, a gaussian smoothing is performed on the overall simulated tomogram volume using the MATLAB "smooth3" routine and with experimentally tuned parameters $\sigma = 51$ and $size = 13$. Finally, the resulting values of simulated RI where re-scaled to the original range of $[1.334, 1.410]$. Fig. 4.2 shows an example of a realistic-looking simulated tomogram compared to a visualisation of an experimentally acquired monocyte. Subfigures (a1), (a2) show an isolevel visualisation of a simulated tomogram and of an experimental one, while subfigures (b1), (b2) compare a yz slice of the simulated and the real tomograms.

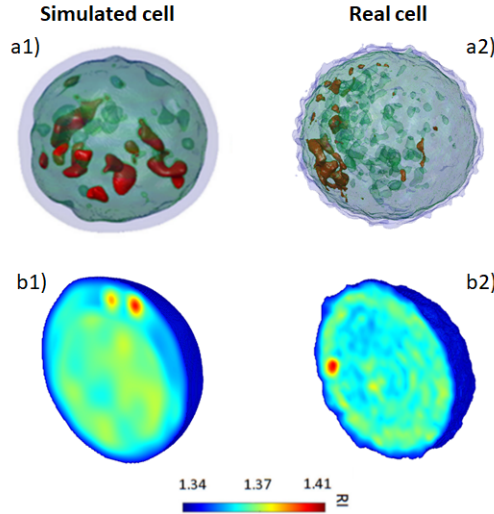


Figure 4.2. Example of a realistic-looking simulated tomogram compared to a real tomogram of a monocyte. (a1), (a2) Isolevel visualisation of the tomograms. (b1), (b2) A yz slice visualisation.

4.3 DL techniques for sparse-view Radon inversion

The problem of inverting the [FST](#) is referred to as Radon inversion and many numerical approaches have been discussed in the previous chapters to handle it. Recently, the idea of incorporating [DCNNs](#) into it has gained a prominent interest, as it allows to achieve reconstruction speed while providing high quality outputs [267, 268]. There are two main strategies to incorporate [DCNNs](#) into the reconstruction pipeline [269]: (i) [DCNNs](#) can be used to perform the Radon inversion process from projection domain to image domain; (ii) [DCNNs](#) can be used as a post processing step made in the image domain to improve the reconstruction quality obtained via [FBP](#). Concerning the case (i), many promising approaches have been proposed. Some of them propose using a [DCNNs](#) to accelerate an iterative process or a subpart of it while preserving the reconstructions' accuracy [270, 271, 272, 273, 274]. Others propose to perform the steps of the [FBP](#) algorithm in a learnable fashion by incorporating [DCNNs](#) [275, 276, 277]. The drawback of using these approaches is linked to the complexity of the transformations from the projection domain to the image domain which the net is supposed to learn [278]; as a consequence, powerful and flexible fully connected layers are preferred to convolutional layers [267]. This indeed improves the reconstruction quality but strongly increases the number of network parameters, with negative effect on the training feasibility and memory occupation. All these approaches, in spite of their remarkable achieved performances, cannot be directly employed into [TPIFC](#) systems. In fact, differently from [ISC](#) systems, in the case of in-flow applications the number of recorded orientations of a rotating cell could not be fixed a-priori [211]. As a consequence, the dimension of the input sinograms in the projection domain cannot be predetermined as the learning of a transformation between the projection domain (mapped into the sinogram) and the image domain would require. Hence decimation or padding operations would have to be carried out to conform to one specifically chosen input size with the potential risk of losing information or adding artefacts. Summing up, for [TPIFC](#), the case (ii) is more suitable and directly applicable, therefore we focus on developing a [DCNN](#) based strategy belonging to this class of methods. Up until now, various methods have been suggested

that perform the Radon transform task by first generating a basic estimate of the solution through FBP, followed by refinement stage obtained using DL. These methods have proven successful in the domain of CT for tackling issues related to low-dose and/or sparse-view scenarios. Indeed, a great research effort is put into lowering the doses of ionising radiation that a patient has to withstand during a CT examination. This can be done either reducing the number of orientations probed (LAT problem) or reducing the power of the x-radiation beam, leading to a worse quality of the recorded input data (low-dose CT). Both these issues are present in LOC oriented solutions for IFC-TPM, and in particular the one discussed in this thesis elaborate in Chapter 3, therefore the outstanding research advancement obtained for CT can provide interesting tools. Concerning this post-FBP refinement step, the most commonly utilised architecture is the UNET DCNN [279, 280]. In [281] and [282], the low-dose CT challenge was approached as an image denoising task solved through UNET. The authors of [283] employed a UNET to learn how to replicate the anomalies introduced by FBP in cases of sparse-view CT in order to subtract them from the initial reconstruction to obtain an artefact-free result. In [278], a UNET was trained to generate a high-resolution reconstruction from a sparsely viewed one. Recent alternatives have been put forth, such as utilising Generative Adversarial Network (GAN)s [284, 285], or employing a combination of DenseNet and deconvolution-based network [286]. However, these approaches often exhibit limited generalisation capabilities due to network instability [287]. As previously mentioned, a critical factor in choosing a network architecture is the number of trainable parameters it has, which directly impacts feasibility of training, memory usage, and reconstruction time. UNET and GAN models typically possess millions of parameters, which can be a significant drawback for imaging cells in the presented high-throuput LOC scenario. Recently, a new network structure called CAN [288] has gained attention due to its distinct approach in replacing the decoder-encoder stages of UNETs, which are usually associated with a decrease in output resolution. In CANs, the spatial dimensions of the input images remain unchanged throughout every layer, while contextual information is integrated by the means of dilated convolutions. Additionally, the CANs network architecture is more straightforward as it does not rely on global skip connections and may allow for a substantial

reduction in the number of parameters compared to UNETs. In [208], the capabilities of CANs were explored in relation to TPIFC, utilising them to speed up the holographic reconstruction process and demonstrating notable outcomes in terms of memory usage and reconstruction speed. Here, CAN is used for the first time to tackle the issue of Radon inversion in recovering the 3D RI distribution of two cell lines, specifically monocytes THP-1 and mouse embryonic fibroblasts NIH-3T3. The experiments were conducted using the setup presented in Chapter 3. We trained the CAN to generate high-quality TPIFC reconstructions from raw FBP outputs utilising a completely synthetic training set, and showcasing the model’s ability to generalise on experimental tomographic data. Specifically, we utilised the numerical cell phantom described in detail in Chapter 5 to generate a range of RI distributions necessary to create a reliable training dataset. The possibility to rely on a fully synthetic dataset is a significant advantage, as the DCNN is trained to replicate what is considered the ground truth of the process, rather than reproducing the output of a reconstruction, which is an approximation. We compare the performance of the proposed CAN-based Radon inversion with conventional FBP, HOTV, and UNET-based approaches, demonstrating its quicker computational speed while drastically reducing the number of adaptable parameters and maintaining reconstruction quality.

4.3.1 Dataset creation

As previously anticipated, a primary challenge in developing reconstruction techniques for microbiological applications, particularly those based on data-driven approaches, arises from the absence of a true reference that can serve as a definitive ground truth. Consequently, training DCNNs using an entirely experimental dataset is a complex undertaking. One potential approach is to consider reconstructions obtained from robust solvers, such as HOTV in our case, as the ground truth. However, this approach would only train the network to approximate HOTV reconstructions, which may be affected by numerical artefacts commonly introduced by numerical reconstruction methods. Another option is to employ a purely numerical dataset, which offers the advantage of being able to synthesise datasets of any required size, a significant benefit for DL techniques. Nevertheless, in the case of numerical datasets, since the

ultimate objective is to develop reconstruction methods applicable to experimental data, meticulous effort must be invested in generating synthetic representations that closely resemble what the actual imaging setup would produce. This is crucial for ensuring generalisation to real-world data. Relying solely on simulated data may lead to a reconstruction approach that is not practically deployable. In light of these considerations, we proceed as outlined below:

1. We generate a fully simulated dataset for training the DCNNs. The performance of the proposed DL approaches is quantitatively evaluated using these synthetic images designated as ground truth.
2. We assess the overall performance of the approaches using experimental datasets derived from actual measurements. In this scenario, we qualitatively compare the quality of the resulting tomographic reconstructions with those obtained via HOTV, which was identified in Chapter 3 as the reference solver for our tomographic setup. We refer to this analysis as the proper test stage of the proposed reconstruction approaches, as it simulates a realistic use-case scenario.

The process for creating the fully numerical dataset used in training the DCNN is detailed in Fig. 4.3. At first, according to the model developed in Chapter 5, 500 3D model cells are generated. Subsequently, from each 3D ground truth cell three 2D images are extracted, considering the central and two slightly peripheral slices, distanced of 5 unities from the central one. Using this procedure, a dataset comprising 1500 slices is created. This dataset is further augmented by horizontally and vertically flipping each image, resulting in a total of 4500 elements. This collection of images constitutes the ground truth dataset. Fig. 4.3 provides an example of a ground truth slice. As mentioned, the DCNNs are trained with a pair of images as input: one being the sparse-view FBP reconstruction, and the other being the corresponding ground truth image. Therefore, starting from the ground truth dataset, the FBP sparse-view dataset must be generated. This is accomplished by reprojecting the ground truth 2D images along specific angular sequences to obtain a sparse-view sinogram using the MATLAB *radon* routine. As thoroughly discussed, in the case of in-flow tomography the illumination angle constitutes an unknown in

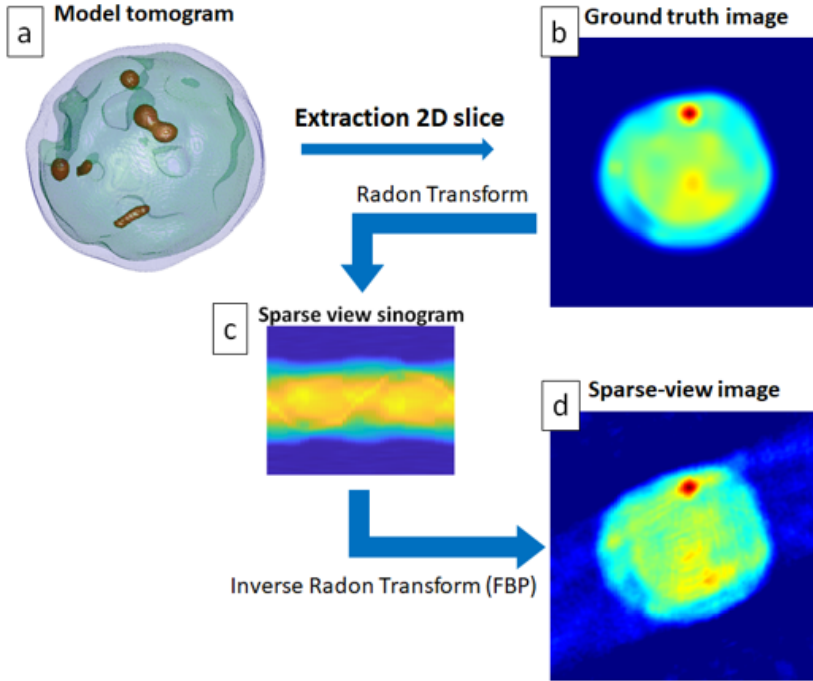


Figure 4.3. Sketch of the procedure followed to create the fully simulated datasets. (a) Isolevel visualisation of a model tomogram of a cell. (b) Ground truth image as the central slice extracted from the model tomogram in (a). (c) Is the sparse-view sinogram obtained by reprojecting the ground truth image in (b) along under-sampled angular sequence. Finally, (d) is the reconstructed slice (sparse-view image) obtained from the sinogram in (c) through the FBP algorithm. Image reproduced from reference [289].

the problem and must be numerically determined afterwards [211]. Additionally, the rotational speed of the cell in the channel is not constant and depends on the cell's position within the FOV [290]. These factors can lead to inaccuracies in retrieving the projection angle, prompting us to model the angular sequence chosen for the reprojection as described by Eq. 4.1 and Eq. 4.2.

$$\begin{cases} \theta_1 = 0 \\ \theta_{i+1} = \theta_i + \text{step}, \quad 1 \leq i \leq N, \quad N.s.t. \theta_N \leq 360 \end{cases} \quad (4.1)$$

$$\begin{cases} \text{Train1} : \text{step} = \text{rand}(0, 5) \\ \text{Train2} : \text{step} = \text{rand}(0, 10) \\ \text{Train3} : \text{step} = \text{rand}(0, 15) \end{cases} \quad (4.2)$$

The first angle of the sequence, denoted as θ_1 , is arbitrarily set to 0. The subsequent angles in the sequence, denoted as θ_i with $i > 1$, are obtained by adding a step to the previous angle θ_{i-1} . The angular sequence is limited to N elements, ensuring that the final angle, θ_N , is less than or equal to 360 degrees. The step used to generate consecutive angles is randomly chosen from a uniform distribution within a specific range (Eq. 4.2). The training process involves updating the network weights in successive stages, gradually addressing a numerically challenging problem, as outlined in [278]. In the first training stage, referred to as "Train 1", the angular step is selected between 0 and 5 degrees. In the second stage, known as "Train 2", the step is chosen between 0 and 10 degrees. In the third stage, named "Train 3", the step varies between 0 and 15 degrees. "Train 1" typically results in 130 projection angles, "Train 2" in 60 projection angles, and "Train 3" in 40 projection angles, which aligns with the realistic number of projections available for the given setup. Employing a random step helps replicating the errors in estimating the projection angle and avoids introducing bias into the learning process. In addition, the overlapping distributions of the angular steps allow the network to be exposed to the same problem with a different degree of complexity, rather than to a different problem. For each ground truth image, a specific angular sequence for tomography is determined and used to reproject it into the measurement domain. The described procedure yields the sparse-view

sinograms, as shown in Fig. 4.3. For each training stage (Train 1, 2, and 3), the training process is conducted in two phases: first without adding noise to the sinograms and then by corrupting them with noise while updating the network weights. The noise is modelled as a normal distribution with the same dimensions as the image and is added to the sinogram. The standard deviation value σ_{noise} of the noise distribution is randomly selected for each cell within a range between 0.1 and 0.25 to avoid biasing the network with specific noise values.

Finally, the FBP reconstructions are generated from the sinograms using the MATLAB *iradon* routine. The resulting 2D FBP reconstruction, depicted in Fig. 4.3, serves as input data during the DCNN training. In total, six sparse-view FBP datasets are created, each corresponding to a specific training stage:

1. "Dataset Train 1": Angular step between 0 and 5 degrees with no noise in the sinogram.
2. "Dataset Train 1 + noise": Angular step between 0 and 5 degrees with noise added to the sinogram.
3. "Dataset Train 2": Angular step between 0 and 10 degrees with no noise in the sinogram.
4. "Dataset Train 2 + noise": Angular step between 0 and 10 degrees with noise added to the sinogram.
5. "Dataset Train 3": Angular step between 0 and 15 degrees with no noise in the sinogram.
6. "Dataset Train 3 + noise": Angular step between 0 and 15 degrees with noise added to the sinogram.

After creating the datasets, a numerical preprocessing stage is applied to each element to prepare the data for DCNN training, referred to as "network preprocessing". Initially, a binary mask is employed to set negative values to zero, excluding those related to the physical process (the retrieval of the always positive RI) but stemming from numerical errors. Subsequently, the values are normalised between 0 and 1 to prevent gradient explosion during training. The training process proceeds sequentially,

updating and refining the DCNN weights using the six datasets created. The network is considered ready for deployment at the conclusion of the "Train 3 + noise" stage. Finally, each dataset is divided into a training set (90% of the total elements in the dataset), a validation set (5% of the total elements), and a test set, which also accounts for 5% of the total according to [278]. It is worth noting that in this training procedure no K-fold cross validation procedure was employed. This choice was mainly due to two reasons: first, the datasets used were sufficiently large and informative enough about the problem; the second reason is linked to the way the simulated model was constructed, lowering the risk of choosing partially informative subsets for the training. In addition, K-folding is often employed to lower the risk of overfitting, and as it will be demonstrated in the next paragraph, in our case the model was not suffering from overfitting. These reasons constitute another advantage of training using simulated data compared to experimental ones, as the entire process is more robust to experimental-dependent particularities. The training process is executed using the Kaggle online platform [291] provided by Google, which offers access to GPUs for computational acceleration. The experimental dataset used to test the learned DCNN models consists of tomographic data from two cell lines analysed in Chapter 3: monocytes THP-1 and mouse embryonic fibroblasts NIH-3T3.

4.3.2 Radon inversion via Context Aggregation Networks

In Section 2.3.3, CANs have been introduced for the automated reconstruction of the holographic processing and the brilliant results it gave in terms of reconstruction speed have been highlighted. Here, for the first time, CAN is used to accelerate Radon inversion computations. CANs have gained attention in the field of image segmentation as an alternative to UNETs [288]. As implied by the name, they aggregate contextual information across increasingly larger scales within the network's layers through a mathematical operation known as dilated convolution. Dilated convolutions involve increasing the size of the kernel in each layer by a certain factor. This allows for the incorporation of long-range contextual information without reducing the image's spatial dimensions, as is the case in the encoder stage of a UNET. Thus, CANs achieve global information aggregation for high-resolution images with a compact parameterisation. In our

work, we implemented the architecture proposed by [292] and adapted it to our specific problem. The CAN network comprises seven layers, with each layer producing 16 feature maps. For the first six layers, the dilation factor of the convolutions increases exponentially by a factor of two, starting from the first layer with no dilation factor applied. Each layer is divided into two branches: one branch propagates the signal as it is with a layer scaling its strength (λ -normalisation layer), while the other branch subjects the signal to Batch Normalization (BN) and an additional trainable sub-layer that adjusts the strength of the BN (μ -normalisation layer). The outputs of the two branches are then added. At the end of each layer, there is a Leaky Rectified Linear Unit (ReLU) non-linearity with a factor of 0.2. The seventh layer is identical to the first layer but without any dilation factor applied to the convolutions. After this layer, a dropout layer with a rate of 0.5 is added, followed by another Leaky ReLU non-linearity. The final stage consists of a single convolution that reports the output in the initial image domain. Fig. 4.4 (a) provides a sketch of the CAN network architecture, while Fig. 4.4 (b) and (c) offer insights into the layout of the layers. The performance of the proposed CAN model is compared with the UNET [279]. Here, we use an architecture inspired by the one in [278] and optimise it for our specific problem. Fig. 4.4 (d) shows the initial Learning Rate (LR) and BS values used for each training stage, along with the number of trainable parameters for both UNET and CAN. We use the Mean Absolute Error (MAE) as the loss function, optimised through the Adam optimiser. The initial value of the LR was chosen empirically to achieve good loss minimisation in the initial epochs. A dynamic monitoring of the LR value is employed to reduce it by a factor of ten whenever the updates in the loss values are smaller than a factor of $1e - 4$ in the last five epochs. The BS value was set as high as possible while considering the memory constraints imposed by the architecture in use. The training procedure is halted without reaching the predefined number of epochs if the updates on the validation loss are smaller than a factor of $1e - 4$ in the last ten epochs. This technique helps prevent overfitting by stopping the training before it reaches a point of stagnation or starts to deteriorate. It is worth noting that, with the same hardware and image dimensions, a larger BS was possible for CAN due to its lighter memory footprint. Both networks were trained using the Kaggle environ-

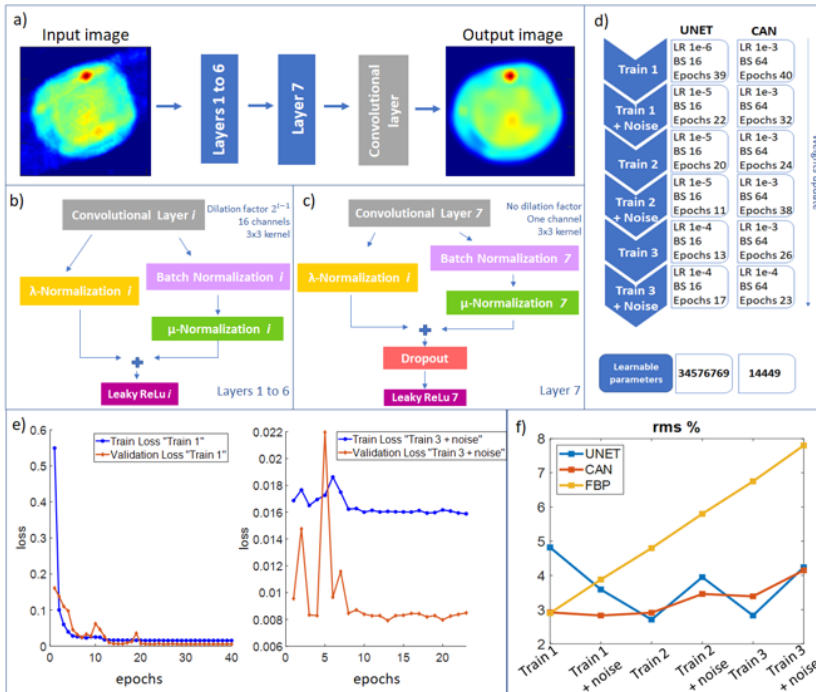


Figure 4.4. Sketch of the implemented training procedure. (a) Schematic view of CAN architecture. (b) and (c) are the inner layout of the layers. (d) Displays the scheme of the training strategy described in Section 4.3.1, with details on the training parameters and number of learnable parameters of the DCNNs. (e) Reports the learning curves for CAN trainings “Train 1” (left) and “Train 3 + noise” (right). (f) Reports the rms% averaged on the elements of the test set. Image reproduced from reference [289].

ment [290] and the GPU Tesla P100 provided by Google. Fig. 4.4 (d) also outlines the workflow of the adopted training strategy for both UNET and CAN, including the progressive weight updates through the various training stages mentioned earlier, as well as the total number of learnable parameters in the DCNN. 4.4 (e) shows the training and validation loss curves for CAN during the "Train 1" stage (left) and "Train 3" + noise stage (right). These loss values indicate consistent learning during the first training stage and fine-tuning during the last stage. The validation loss curve suggests robustness against overfitting. Finally, Fig. 4.4 (f) presents the rms% averaged across the 225 items in the test set for each training stage. Due to its higher number of parameters, UNET is unable to provide high-quality results after just one training stage and introduces numerical artefacts in the reconstructions. Conversely, after one stage of training, CAN produces results of comparable quality to the FBP method. As the number of projections decreases and noise is introduced, the FBP method exhibits consistently increasing rms% values, while the networks offer a significant improvement in comparison. After the last training stage, CAN and UNET provide reconstructions of comparable quality. In the presence of noise, CAN reconstructions generally exhibit greater accuracy compared to UNET, as demonstrated by the mean results over the entire simulated test set.

4.3.3 Inference results for simulated data

	CPU time [s]	rms%, total area	rms%, cell region
FBP	0.2	20.43	16.37
UNET	1.68	6.18	6.58
CAN	0.38	7.11	8.80
HOTV2	10.10	2.93	4.03

Table 4.1. Comparison between the reconstruction times needed using FBP, HOTV2, UNET and CAN to reconstruct the images of Fig. 4.5.

A first testing evaluation is conducted still on simulated data, before moving on the testing stage with experimental data. Three new simulated FBP images were generated corresponding to "Train 1 + noise", "Train 2 + noise", and "Train 3 + noise", using a new simulated cell. Fig. 4.5 presents

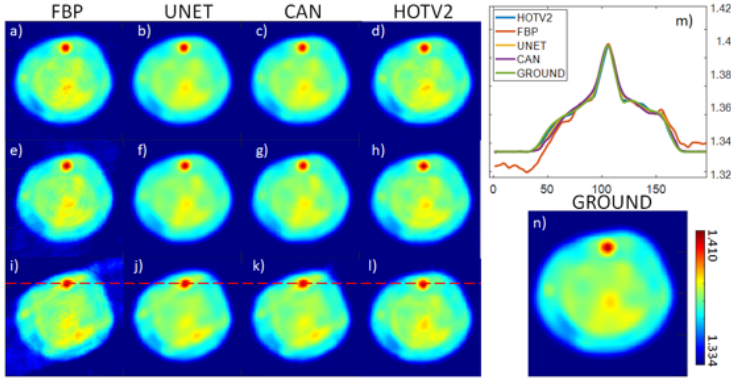


Figure 4.5. Comparison between different reconstruction algorithms (FBP, UNET, CAN, HOTV2) for "Train 1 + noise" (a-d), "Train 2 + noise" (e-h), "Train 3 + noise" (i-l). (n) shows the ground truth image. Finally, (m) reports the plot of a single horizontal line of (i), (j), (k), (l) over the dashed red line. Image reproduced from reference [289].

a comparison of the reconstructions obtained with different solvers (FBP, UNET, CAN, HOTV2) for these images, alongside the ground truth reference (Fig. 4.5 n). As previously discussed, the FBP reconstructions (Fig. 4.5 (a, e, i)) exhibit a noticeable amount of background noise and internal artefacts. The problem of artefacts is linked to the ramp filtering operation performed by the FBP algorithm over the projections, which tends to enhance noise present in the raw signal primarily at high spatial frequencies, like borders and boundaries. These noise contributions, peculiar of the FBP, are therefore visible over the reconstruction. UNET (Fig. 4.5 (b, f, j)) and CAN (Fig. 4.5 (c, g, k)) outperforms FBP significantly in terms of both quantitative and qualitative performance, improving the background and partially addressing internal artefacts. As expected, HOTV2 (Fig. 4.5 (d, h, l)) produces the highest quality reconstructions in terms of shape retrieval and artefact suppression. The plot in Fig. 4.5 (m) highlights the beneficial effect of background removal performed by the DCNN compared to FBP. Both UNET and CAN contribute to reducing the artefacts in the internal regions introduced by FBP. Generally, the CAN reconstruction exhibits a smoother profile compared to UNET, which tends to introduce more noise. This difference is attributed to the varying complexity of

the two network models and the higher number of parameters in UNET. Table 4.1 provides the CPU times required to obtain the reconstructions depicted in Fig. 4.5 (i, j, k, l) using a commercial desktop computer. The total reconstruction time for both CAN and UNET is calculated as the sum of the time needed to compute the FBP reconstruction and the actual processing time of the networks. The FBP algorithm is notably fast, while the iterative solver increases the reconstruction time by about two orders of magnitude. In contrast, the DCNNs are generally over one order of magnitude faster than the iterative solver. Specifically, the CAN network processes the FBP reconstruction in approximately one-third of the time required by the UNET. This efficiency difference can be attributed to the lighter architecture of the CAN network, which has 15,000 parameters compared to the UNET's 35 million (as shown in Fig. 4.4(d)).

To provide a comprehensive evaluation, Table 4.1 also presents the rms% between the reconstructions obtained by different solvers and the ground truth image for the test images depicted in Fig. 4.5 (i, j, k, l). The rms% is calculated for both the entire slice area (including both the background and the internal cell region) and the cell region alone. The DCNNs demonstrate a significant advantage, particularly in the background area, and their impact is also beneficial compared to the FBP in the internal cell region. In summary, the HOTV2 solver outperforms other methods in terms of reconstruction accuracy. The FBP introduces significant corruption in both the internal cell region and the background area. The CAN and UNET predictions offer comparable reconstruction quality. In conclusion, both DCNNs achieve the initial objective of obtaining reconstructions of improved and comparable quality compared to the FBP. However, the UNET is considerably slower in deployment compared to the CAN. This confirms that reconstruction approaches based on CAN architectures are well-suited for applications where the primary goal is to achieve high throughput and faster processing, rather than aiming for extremely accurate reconstructions.

4.3.3.1 Assessment of the training strategy

For the training of the DCNNs, we resorted to the described unconventional training strategy, inspired by [278] and based on a transfer learning approach, aiming to enhance training effectiveness and produce a more

robust model for handling experimental data. Indeed, it is worth again pointing out the reconstruction problem we are tackling: the network in deployment must be able to reconstruct FBP data obtained considering a non-constant angular step ranging between 0 and 15 degrees and in presence of noise over the measured data (the sinogram) introduced by the coherent nature of the optical system. This task is a complex task and transfer learning approaches are often useful to help approaching it. However, complex training models might result in problems like overfitting which mine the ability of the networks to be deployed in real-life scenarios. Hence, to prove the benefits it delivers, different training strategies have been explored. In particular:

1. We trained the networks considering a unique dataset corresponding to the one termed “Train 3” in the Section 4.3.1. In this dataset, the FBP reconstruction are obtained using directly the angular step corresponding to the experimental case, so ranging between 0 and 15° , and without adding noise to the sinograms. In this case, no pretrained network weights were loaded into the network.
2. We trained the networks considering a 1:5 decimated version of the full dataset described in the Section 4.3.1 and termed “Train 3 + noise”, i.e. by considering only 810 elements in the training dataset. No pretrained network weights were loaded into the network.

Fig. 4.6 illustrates the results for the training approach described in point 1). Specifically, Fig. 4.6 (a1) displays the FBP data used as input for the networks, while Fig. 4.6 (a6) presents the ground truth reference image. Fig. 4.6 (a2) and Fig. 4.6 (a4) show the predictions generated by CAN and UNET, respectively, and Fig. 4.6 (a3) and Fig. 4.6 (a5) depict the reference CAN and UNET reconstructions at the “Train 3” stage, representing the transfer learning approach. As indicated by the red arrows, without using transfer learning, the CAN network struggles to reconstruct finer structures. Conversely, UNET can achieve better results than CAN even with this type of training. However, analysing the learning curves for CAN (left side of Fig. 4.6 (b)) and UNET (right side of Fig. 4.6 (b)), it becomes evident that UNET is prone to overfitting, which negatively impacts its generalisation performance. Table 4.2 provides the rms% values calculated across the entire test set for CAN and UNET trained with the

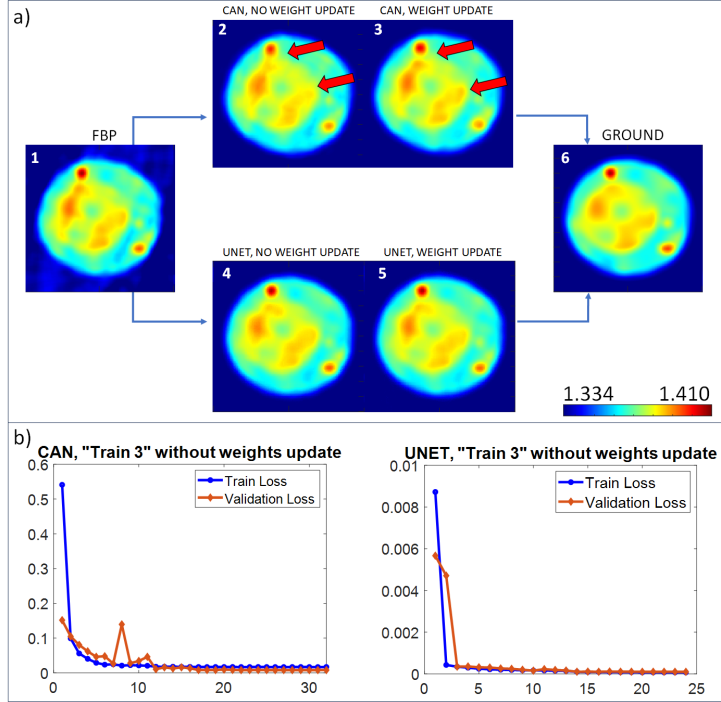


Figure 4.6. Results for networks training without the use of transfer learning. (a1) Input FBP image; (a2-4) CAN and UNET reconstruction for the mentioned training; (a3-5) CAN and UNET reconstructions for the training making use of transfer learning; (a6) ground truth image. (b) Learning curves for CAN (left) and UNET (right) for the training without transfer learning. Image reproduced from reference [289].

mentioned dataset. Additionally, it includes the rms% values obtained using the transfer learning approach for comparison. The rms% values affirm that the transfer learning approach yields a benefit for the CAN training procedure.

Trained model	rms%
CAN without transfer learning	4.03
CAN with transfer learning	3.39
UNET without transfer learning	2.64
UNET with transfer learning	2.83

Table 4.2. Comparison between different training strategies for the networks. Incorporating transfer learning into the training allows better results.

Fig. 4.7 illustrates the results of training with a decimated dataset at a factor of 1:5. Specifically, Fig. 4.7 (a1) displays the FBP input image, while Fig. 4.7 (a4) presents the ground truth reference image. Fig. 4.7 (a3) and Fig. 4.7 (a4) showcase the predictions obtained with CAN and UNET, respectively. It is important to note that CAN struggles to accurately predict the internal structures of the cell, while UNET, although visually appearing to perform better, provides a numerically unstable reconstruction in the border area. In contrast, CAN consistently produces a smooth reconstruction. This difference in performance may be attributed to the varying number of parameters that need to be trained in the two architectures. Analysing the learning curves in Fig. 4.7 (b), it becomes evident that both models suffer from overfitting, with UNET exhibiting a stronger degree of overfitting, as supported by the loss values. Table 4.3 reports the rms% values calculated across the entire test set for both the full and decimated datasets. In both cases, a significant reduction in overall performance is observed when using the decimated dataset. These results underscore the challenges associated with training using a decimated dataset, which leads to reduced performance and overfitting issues for both CAN and UNET, particularly in the case of UNET.

	rms%, full dataset	rms%, 1:5 decimated dataset
CAN	4.15	22.52
UNET	4.24	18.09

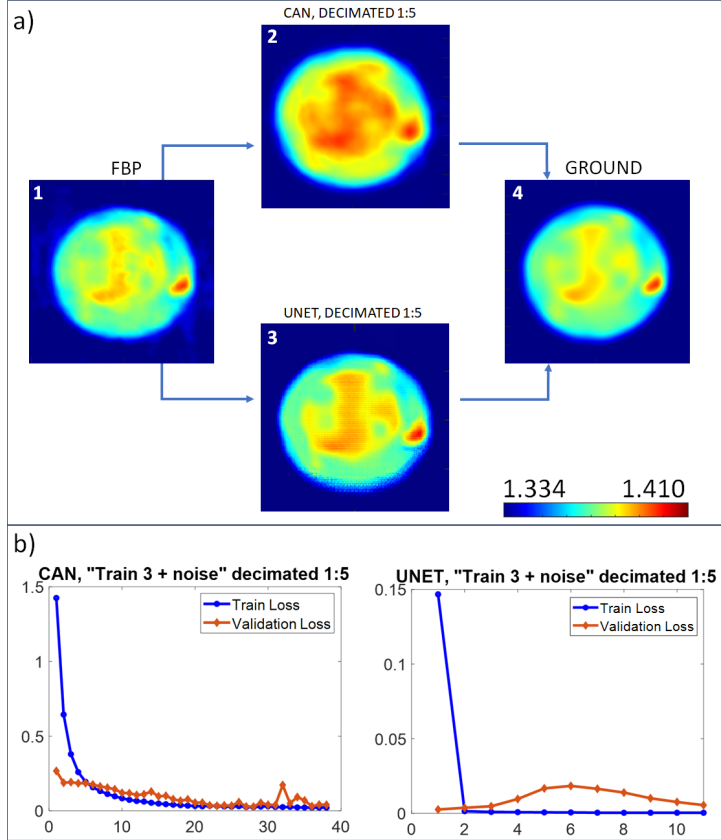


Figure 4.7. Results for networks training considering a decimated version of the dataset “Train 3 + Noise” of a factor 1:5 and no transfer learning. (a1) input FBP image. (a2-3) CAN and UNET reconstruction for the mentioned training. a4) Ground truth image. (b) Learning curves for CAN (left) and UNET (right).

Table 4.3. Results for the training of the networks considering a decimated dataset.

4.3.3.2 Generalisation capabilities of the trained model

As previously mentioned, the entire reliability of the training process is linked to the reliability of the cell modelling underneath, as the quality of the dataset directly impacts the network’s ability to effectively reconstruct experimental data. However, as discussed, the correct modelling of biological samples remain a challenging task in microscopic imaging due to the lack of an universally recognised ground truth. While the synthetic dataset discussed in this study has demonstrated a high level of realism [293], certain assumptions were made when creating the model cell used as ground truth. The **RI** values used to model the internal structures of the cells were drawn from Gaussian distributions, and the noise used to corrupt the sinograms was modelled as additive Gaussian noise, simulating the effects of the global point spread function of the optical system. These assumptions were inspired by experimental data, as reported in [263, 208, 293]. To further demonstrate the robustness of the developed reconstruction approach using **DCNNs**, the trained networks were tested on simulated data with **RI** values drawn from a uniform distribution, and speckle noise was introduced to corrupt the sinograms. Fig. 4.8 presents the results of these analyses: Panel 4.8 (1) displays (from left to right) the **FBP** reconstruction, the predictions of **UNET** and **CAN**, and the ground truth image of a simulated cell with a uniform distribution for the **RI** values. The **RI** values have an average value of 0 and a standard deviation of 0.2 for the mitochondria and 0.5 for the other internal structures simulated. Both networks can still produce a smooth reconstruction, removing background artefacts and minimising internal ones, demonstrating their generalisation capabilities over this type of test data. The corresponding rms values in percentage, computed between the reconstructions and the ground truth, are reported at the bottom, indicating performance comparable to that on the simulated test set with additive Gaussian noise, as shown in Table 4.3. Panel 4.8 (2.1) instead demonstrates the effect of speckle noise on the sinograms (left) in comparison with additive Gaussian noise (middle) and uncorrupted data (right). Speckle noise significantly degrades the sinogram and generates ring-shaped artefacts into the **FBP** reconstruction, as

visible in Panel 4.8 (2.2). For FBP reconstructions, a random angular step between 0 and 5° is used as a demonstrative case. Again, the networks exhibit great stability even in this highly perturbed case, confirming their generalisation capabilities. The performance of the networks is similar to the results obtained with Gaussian noise, although slightly degraded.

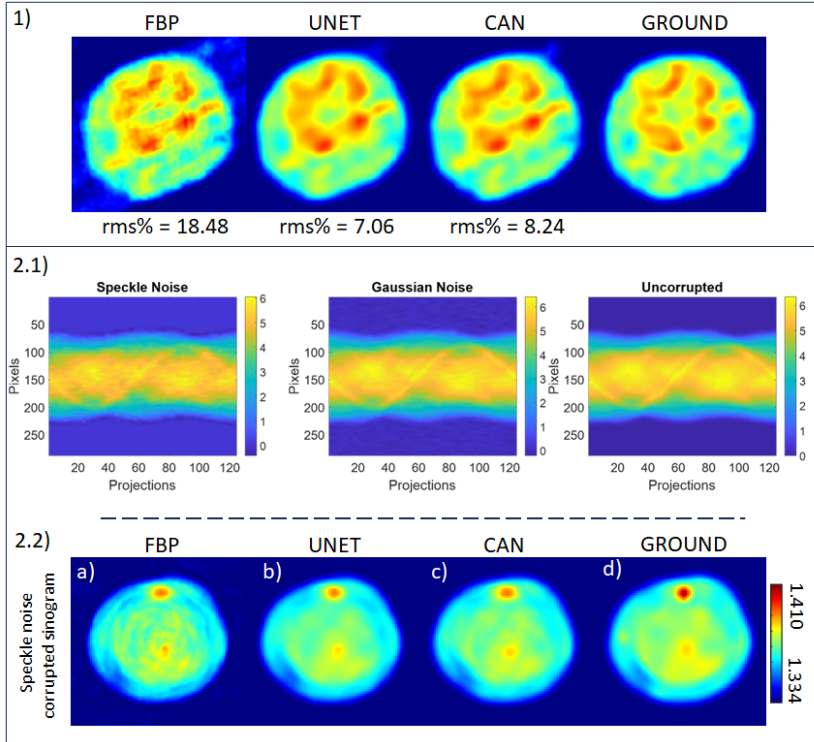


Figure 4.8. Subpanel 1: results considering RI values drawn from an uniform distribution. Subpanel 2.1: comparison between different types of noise applied over the sinogram. Subpanel 2.2: results for UNET and CAN in the case of speckle noise added over the sinogram for an angular step between 0 and 5° . Images (a) FBP reconstruction. (b) UNET reconstruction. (c) CAN reconstruction. (d) Ground truth. Image reproduced from reference [289].

Table 4.4 reports the rms% values computed between the reconstructions and the ground truth for the reported image and two other cases where the FBP images were computed with random angular steps between

0 and 10° and between 0 and 15° . These rms values further confirm the robustness of the network reconstructions in the presence of speckle noise sinogram corruption, even for these cases.

Random angular step interval	FBP	UNET	CAN
$[0, 5^\circ]$	14.41	12.15	8.79
$[0, 10^\circ]$	17.47	10.45	10.73
$[0, 15^\circ]$	24.98	11.96	12.63

Table 4.4. rms% computed between the reconstruction in Fig. 4.8, bottom, obtained for speckle-noise corrupted sinograms, and the ground truth for different angular step intervals.

4.3.4 Experimental test set results

The quantitative assessment over simulated data provides a foundation for deploying the networks on experimental test images reconstructed via FBP. Fig. 4.9 presents the results for four different THP-1 monocytes (left, Fig. 4.9 (a-d)) and four NIH-3T3 mouse cells (right, Fig. 4.9 (e-h)) for a central slice, computed with FBP, UNET, CAN, and HOTV2. As observed, the background-noise reduction effect observed in simulated data is also evident in real data. Both CAN and UNET yield to predictions that are highly consistent with each other, introducing a smoothing effect on the internal structures of cells, with this effect being more noticeable in the reconstructions obtained via the UNET network. For quantitative evaluation, Table 4.5 reports the rms% values, averaged over the 42 examined monocytes, between the central slice reconstructed by the investigated methods and the HOTV2 results, which serve as the reference method in the absence of ground truth images for experimental data. It is important to note that there may be a reduced accuracy in the predictions of both CAN and UNET when comparing the rms% values in Table 4.1 (simulated data) and Table 4.5 (experimental data). This difference is primarily due to the evaluation criteria. In the case of simulated data, performance can be assessed against an ideal ground truth, whereas the rms% values in Table 4.5 are evaluated using HOTV2 reconstructions as a reference. It is worth mentioning that HOTV2 operates on the FBP as an initial guess and aims to improve it, while both CAN and UNET attempt to predict the ideal

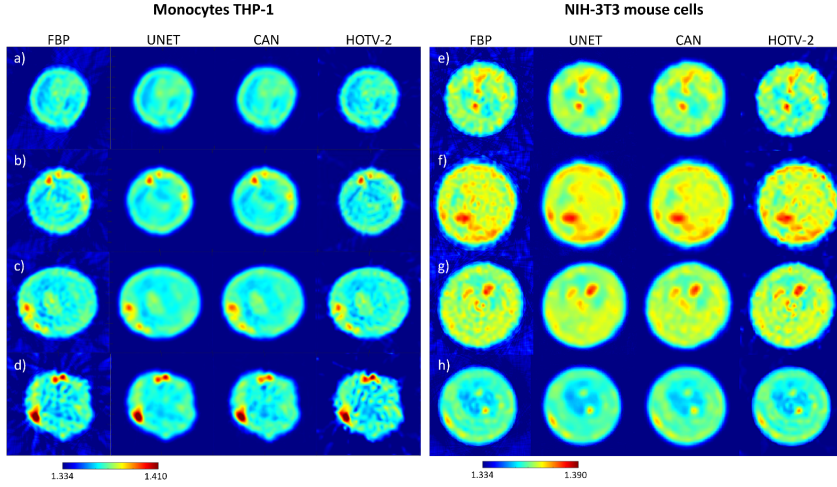


Figure 4.9. Comparison of the central slice reconstructions obtained for monocytes THP-1 (a–d) and NIH-3T3 mouse cells (e–h) with different solvers (FBP, UNET, CAN, HOTV2).

ground truth. This is clearly noticeable in Fig. 4.9, where a great similarity between the HOTV2 reconstruction and the FBP can be appreciated. However, the apparent similarity between the two reconstructions cannot be considered as a direct consequence of a more reliable reconstruction, as no ground truth is available for the case of experimental data. Indeed, quantitative comparison can only be made for the case of simulated data. In this case, using HOTV2 as a designated ground truth may introduce biases in evaluating the rms%, favouring the FBP reconstruction with respect to the networks, which reconstruct using a completely different physical processing compared to HOTV2. In conclusion, the DCNNs, specifically the CAN network, demonstrate similar performance. This confirms that the CAN network provides reconstructions of quality comparable to the state-of-the-art UNET and offers a significant improvement compared to the FBP, particularly in the background region. As a last test case, the ability of the CAN to predict peripheral slices of tomograms is evaluated, even though they were not included in the training stage, as the networks are trained only using two slightly peripheral slices apart from the central one as a ground truth. Fig. 4.10 shows the slice-by-slice reconstruction

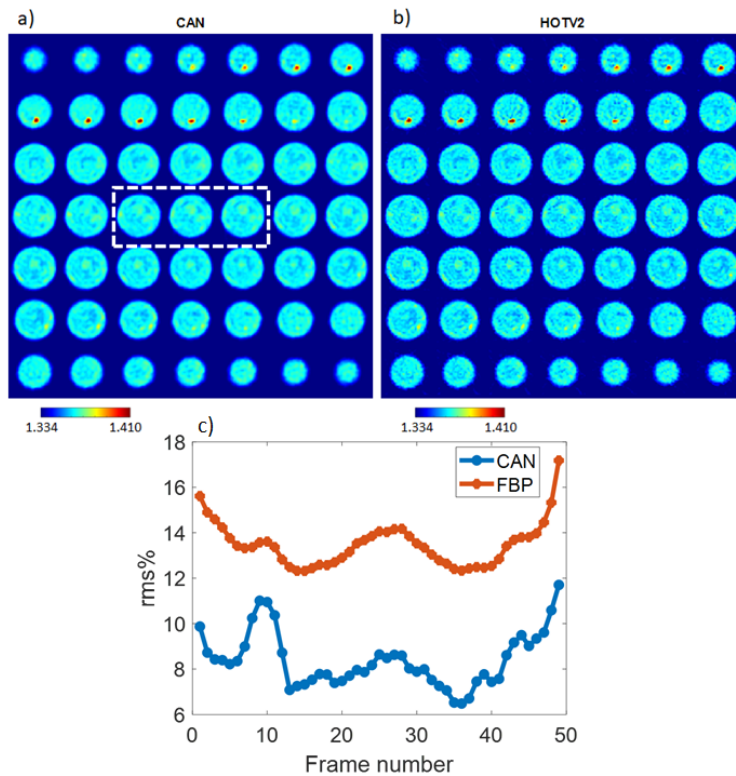


Figure 4.10. Assessment of the generalisation capability of the CAN over peripheral slices never used in the training stage. Slice-by-slice reconstructions with (a) CAN and (b) HOTV2, obtained from a tomogram of a monocyte THP-1, are reported. red

(c) rms% normalised evaluated between CAN and HOTV2 for each displayed frame. As a comparison, the same quantity evaluated for FBP is displayed too.

of a monocyte THP-1 tomogram for **CAN** (Fig. 4.10 (a) and for **HOTV2** (Fig. 4.10 (b), with a focus on the three slices learned by the **CAN** model (indicated by the dashed white box in Fig. 4.10 (a)). Fig. 4.10 (c) reports the value of rms% normalised evaluated between the **CAN** reconstructions and **HOTV2** ones for each displayed slice. As a comparison, the same quantity is reported for the **FBP** reconstruction too. The **CAN** performs accurate predictions in this scenario, as indicated by the average rms between the slices of **HOTV2** and those of **CAN**, which is equal to 8.39%. This test further confirms the generalisation ability of the proposed **CAN** model, which is consistent with the absence of overfitting suggested by the training curves in Fig. 4.10 (3).

	rms%, total area	rms%, cell region
FBP	17.13	14.32
UNET	11.68	14.15
CAN	12.91	15.56

Table 4.5. Reconstruction accuracies for experimental data tests (**HOTV2** as the reference method).

4.3.5 DL-aided 3D tomographic reconstruction

The described technique was meant to reconstruct 2D tomographic data. However, it can be easily extended to 3D data considering the 3D volume a stack of 2D slices. Given the demonstrated successful capabilities of UNET and **CAN** to generalise over peripheral data (Fig. 4.9), this strategy has been used to reconstruct 3D tomograms of **WBC** as in Chapter 3. Fig. 4.11 shows the isolevel visualisation of a 3D tomogram obtained using different solvers, in particular (a) **HOTV2**, (b) **FBP**, (c) UNET, (d) **CAN**. Note that in each case, even for **FBP** and **HOTV**, the reconstruction is obtained stacking 2D slices. The two thresholds chosen for the visualisation are set as 0.3 and 0.6 of the maximum level of **RI** measured over the **HOTV2** reconstruction. The tomographic reconstruction obtained via the network processing of each 2D slide is consistent because the external shape of the cell is preserved, as made evident by the distinctive detail present in the chosen cell which is effectively retrieved. The most peripheral slices constitute a problem for the networks, as the

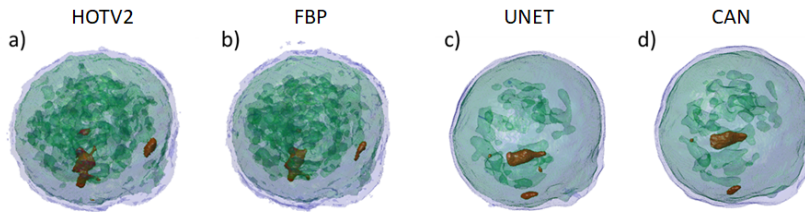


Figure 4.11. Isolevel visualizations of 3D tomograms obtained stacking 2D slice reconstructions. (a), **HOTV**, (b), **FBP**, (c), **UNET**, (d), **CAN**.

information they carry is corrupted by a decisive amount of noise due to diffraction effects and therefore strongly differ from the training data of the network. This can be seen in Fig. 4.12, where in case A, B, C, D the reconstructions at slices 17, 18, 21 and 25 are shown, respectively. However, while **UNET** completely fails to provide a reliable reconstruction, **CAN** produces a more stable output (Fig. 4.12, case A, B). At slice 25, the output of both the networks is correctly formed. For cases C and D, the values of rms% normalised computed between the **FBP**, **UNET** and **CAN** illustrated slices and the corresponding **HOTV2** ones are reported. The mean value of rms% normalised computed for the slices 25 to 175 between each reconstruction and **HOTV2** is equal to 16.39 for **FBP**, 9.14 for **UNET** and 10.33 for **CAN**. The most peripheral slices were excluded from this computations for the noise problems mentioned. Even though some smoothing is added to the tomograms by the networks, a full 200x200x200 tomogram is obtained in circa 45s of CPU time using **CAN** (4.6), versus the 15 minutes needed by the **HOTV2** solver.

	CPU time
FBP	4 [s]
UNET	4.5 [min]
CAN	45 [s]
HOTV2	15 [min]

Table 4.6. Computing times and reconstruction accuracy for simulated data tests.

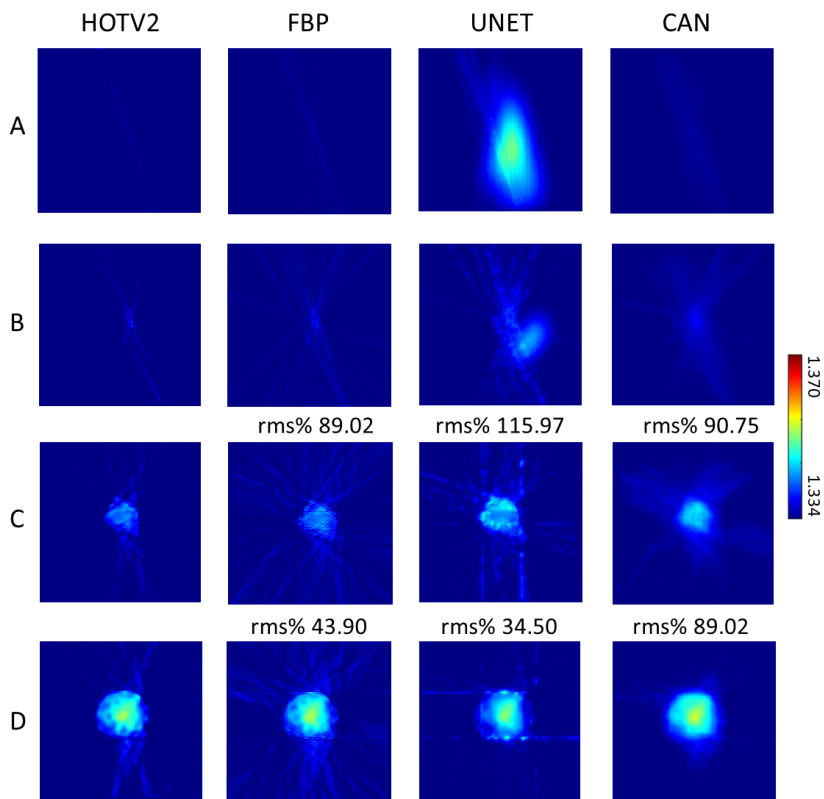


Figure 4.12. Comparison between the performance of the different solvers in the reconstruction of the peripheral slices. Case A, slice 17, B, slice 18, case C, slice 21, case D, slice 25. The values of rms% normalised computed between the depicted slices and the corresponding [HOTV2](#) ones are reported as a comparison.

4.3.5.1 Reconstruction of fully 3D tomographic data

With the aim to extend the previous result, the same strategy can be used to realise entirely 3D approaches, exploiting network architectures operating over volumetric data. This is accomplished adding a third dimension to the input of the networks, to the convolutional kernels and to the pooling stages. However, the limitation in this approach is linked to the exponential augmentation of memory occupation and processing times of the 3D dataset and for the network training. Accordingly, tomograms of size $64 \times 64 \times 64$ were used instead of the experimental $200 \times 200 \times 200$ due to memory limitations. The same procedure used for the training of the 2D data was used for the 3D case. Concerning the dataset used, as in the previous case 1500 model cells of size $200 \times 200 \times 200$ were generated and then resized to the size of $64 \times 64 \times 64$ via bicubic interpolation. Instead of directly generating lower dimensional data, the choice of resizing was made to have consistency with experimental data which have size $200 \times 200 \times 200$ and that have to be resized to be processed by the network. Regarding UNET, the network architecture presents 5 convolutional-max pooling-ReLU blocks for the encoding and decoding stages. The first convolutional layers have 32 activation maps; this number is doubled after each pooling stage. The BS is 16. The chosen LR is equal to $1e-3$ except for the stage "Train 1" in which it was chosen as $1e-5$. For CAN, the BS was fixed to 10 and the LR to $1e-3$. The number of feature maps of the convolutional layers is 16. The number of parameters of the UNET is 25924353, whereas for CAN is 42897. The chosen loss is a combination of Mean Standard Error (MSE) and the MAE in a weight ratio 8:2. The first observed advancement compared to the 3D slice-by-slice reconstruction is the possibility to overcome the issues regarding the peripheral slices. Since the data is processed as a whole unit, as shown in Fig. 4.13, both the networks can successfully produce a resolved output even in the case of extremely peripheral slices. Concerning the reconstruction of real data, Fig. 4.14 shows the central y-z slice of a tomogram of a WBC and the rms% normalised computed for the entire 3D tomograms reconstructed with each solver and the HOTV2 reconstruction. The data has size of $200 \times 200 \times 200$, so the output of the networks have been upscaled to this dimension. The rows from top to bottom show the output of the HOTV2 reconstruction, FBP, UNET and CAN respectively. The same benefits described for the 2D cases can be

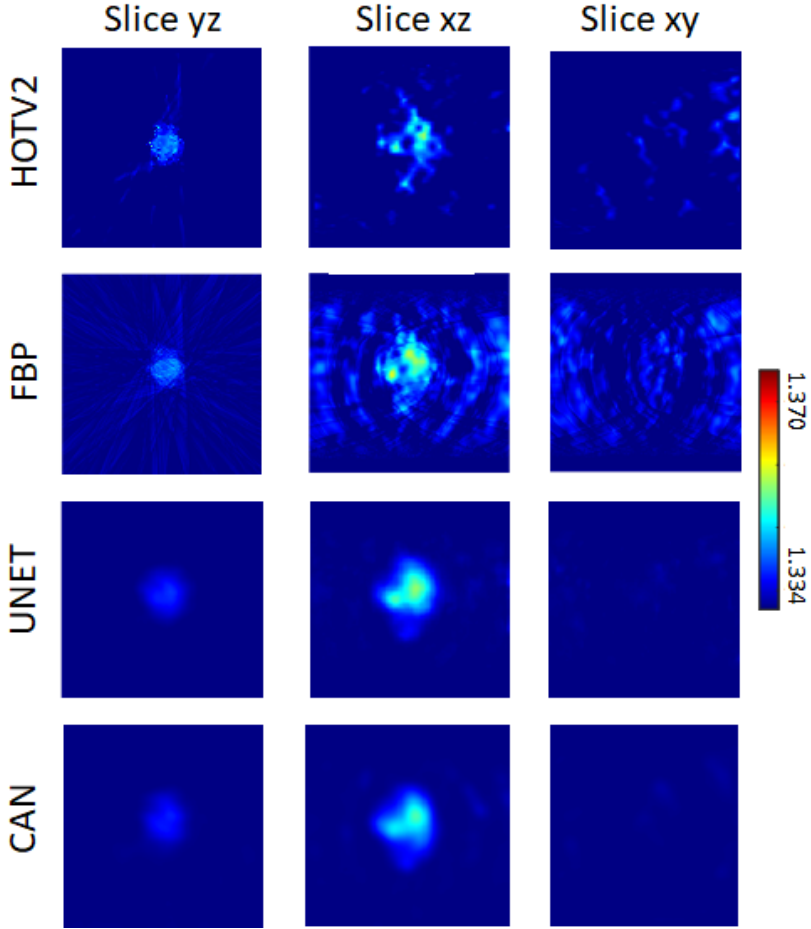


Figure 4.13. Peripheral slice reconstruction considering different solvers for simulate data. From top to bottom: **HOTV2**, **FBP**, **UNET**, **CAN**. **FBP** and **HOTV2** allow to obtain a 3D reconstruction as a stack of 2D slices; the networks, conversely, operate a 3D reconstruction.

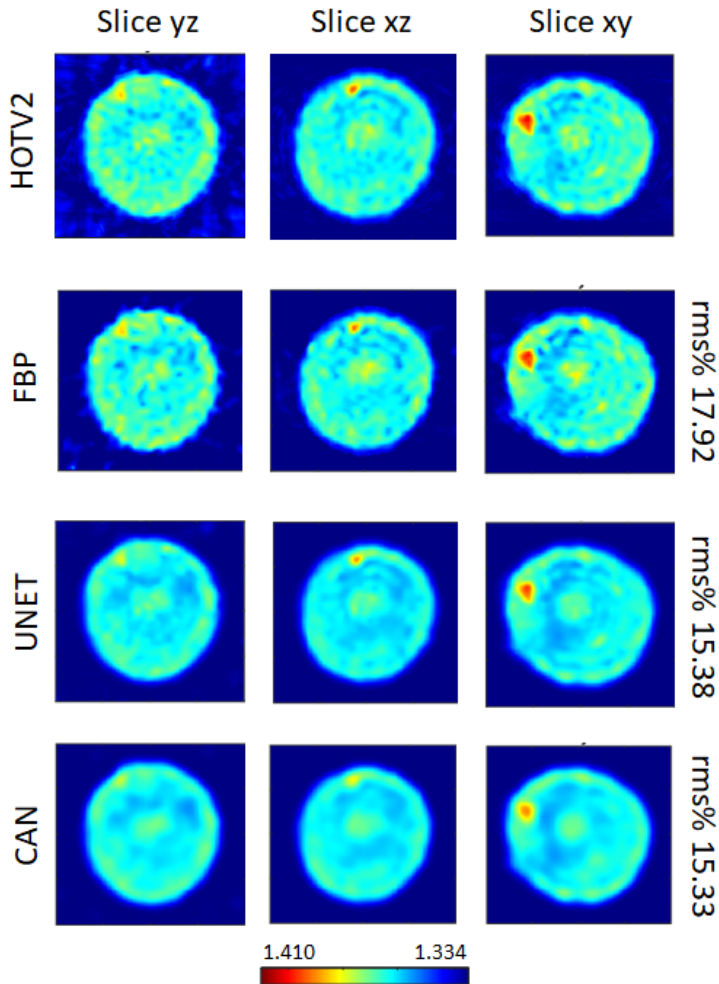


Figure 4.14. Central slice reconstruction using different solvers of an experimentally acquired monocyte. From top to bottom: [HOTV2](#), [FBP](#), [UNET](#), [CAN](#). The values of rms% normalised evaluated between the entire 3D tomograms reconstructed via [FBP](#), [UNET](#), [CAN](#) and the [HOTV2](#) reconstruction are reported.

observed in terms of background replenishment. However, in the case of **CAN**, a stronger smoothing effect is present. The rms% values confirm the improvement brought by the networks. **HOTV2** and **FBP** provide similar reconstructions in the internal area; while the networks, as expected, introduce a smoothing in the internal zone. Nonetheless, the improvement brought by the **CNNs** in the external area is enough to still provide a lower rms. Finally, concerning the CPU reconstruction times for the reconstruction of 64x64x64 data and reported in Table 4.7, a decisive improvement in terms of time can be noted brought by the networks in general, but in particular by **CAN**.

	CPU time [s]
FBP	1
UNET	1 [FBP] + 3.38 [UNET]
CAN	1 [FBP] + 0.2 [CAN]
HOTV2	540

Table 4.7. Computing times for the reconstruction of 3D data.

4.4 DL for super resolution in TPM

Resolution enhancement or Super Resolution (**SR**) for images is defined as the process allowing to obtain an High Resolution (**HR**) image starting from an Low Resolution (**LRE**) acquired one. This eventuality is sought after in many applications such as intelligent surveillance, remote sensing and medical imaging [294], where **LRE** acquisition setups might be desirable for cost reduction, security reasons and to potentially achieve larger spatial coverage and higher signal to noise ratio. Mathematically, the problem can be described as

$$Y = f(X) \quad (4.3)$$

Where Y is the **LRE** image, X the **HR** one and f is an arbitrary function representing the downgrading transformation. Restoring the resolution via an **SR** process means to estimate a function $g = f^{-1}$ such that $X \approx g(Y)$. **SR** techniques can be grouped into two main categories: (i) Video **SR**, where the aim of the **SR** technique is to numerically augment

the framerate of the acquisition, and (ii) Image SR, where the objective is to numerically augment the number of pixels of an image. The class (ii) can be itself grouped into two main subgroups, nominally Multi-Frame Image Super Resolution (MFSR), in which the resolution enhancement is obtained merging contributions coming from different acquisitions of the scenario, and Single Image Super Resolution (SISR), in which a single image is processed to obtain a resolution increase. Although MFSR is a valuable technique, it is really sensitive to inter acquisition motion, which is frequent in medical imaging applications such as MRI and CT scans. Therefore, SISR has emerged as the most widely studied category in the literature and many approaches have been studied. Traditionally, SISR was mainly approached via simplistic methods such as [295, 296, 297, 298, 299] using bilinear or bicubic interpolations [300]. These methods are often chosen as they are characterised by a low computational cost and are general purpose, however, since they do not consider the characteristics of the specific subjects, their simplicity limits their capability to enhance image quality. Moreover, they tend to generate excessively smooth images with artefacts [301] and fail to recover the loss of high frequency information like fine edges of objects. Other simplistic approaches frequently used require to model the SISR problem as an optimisation problem to minimise the cost between observed LRE image and the model estimation, often embedding regularisation terms such as TV [299]. However, these methods are generally limited as they require important prior knowledge about the data representation. More powerful approaches such as utilising statistical image priors [296, 297] or internal patch recurrence [298] can be used as an alternative. Learning methods have been proposed and are used to model a mapping from LRE to HR patches. Neighbour embedding [302, 303] methods interpolate the patch subspace, while sparse coding [304, 305, 306, 307] methods instead use a learned compact dictionary based on sparse signal representation. A comprehensive review of these approaches can be found in [308]. However, the breakthrough in SR methods happened with the advent of DL techniques as it was proven that a neural network can easily model the relation expressed in Eq. 4.3. In the seminal work [308], for the first time a very shallow CNN named SRCNN was proposed, composed of three layers able to learn an end-to-end mapping between the LRE and the HR image. SRCNN provided outstanding benefits in terms of speed and

accuracy compared to the previously employed methods. The introduction of this pioneering work paved the way to the development of a new research branch for [SISR](#) [309]. The authors of [310] demonstrated the beneficial effects of increasing the depth of the network to further increase the accuracy of the reconstruction obtained with SRCNN. In particular, they were able to solve the major issues of SRCNN linked to its extremely high convergence time and lack of flexibility in the choice of the scale of underresolution which had to be determined a-priori. With their proposed network, named VDSR, the authors were able to increase the size of the contextual information used for the specific task, increasing the accuracy. In addition, they were able to increase the value of used [LR](#), speeding up the training procedure. The authors of [311] successfully proposed an improvement to SRCNN aiming to speed up consistently its training and improve its results. The network they proposed, named FSRCNN, was characterised by a compact hourglass-shape, presenting a deconvolution layer at the end of the network, and by a reformulated mapping layer from the [LRE](#) domain to the [HR](#). Finally, they introduced a bigger number of mapping layer of smaller size. Concluding, the authors of [312] perfected even more the results obtained with the VDSR by using a slimmed-up version of a SRResNet, introduced by [313], from which unnecessary layers had been removed. This simplified network architecture, named EDSR, is simpler to train than other models and allows to obtain a lighter computational burden. In addition, the authors proved the advantages of using a l_1 -norm compared to an l_2 one into the loss function. [SISR](#) has been successfully integrated into medical imaging resolution enhancement. In [314], the authors applied SRCNN to low resolution [CT](#) demonstrating its ability to outperform traditional linear interpolation methods. In [315], the authors proposed the use of the UNET architecture for [SR](#) problems, whereas in [316, 317] [GAN](#)-based nets were used for 2D [CT](#) scan super resolution. However, all the mentioned approaches [SISR](#) and in particular their application to medical image reconstruction are meant for 2D reconstructions. The problem of applications to 3D imaging remains open, as the methods developed for 2D imaging cannot be directly translated into the 3D domain given the enormously greater computational load they provide and the fact that considering a 3D domain as a stack of 2D slices has the inherent mentioned problem of misalignment artefacts and neglect-

ing the 3D nature of the problem without taking advantage of continuous structures in 3D. In [318] the authors discussed the possibility of employing 3D SR model for MRI images. In [319], the authors discussed the difference into utilising the 2D SRCNN applied slice by slice for a 3D problem versus employing a 3D version of SRCNN, concluding that for an intrinsically 3D model such as medical 3D imaging 3D solutions always yield to better results. In [320] the authors applied a network similar to a 3D version of the 3DSRCNN for the SR of cardiac images, while the authors of [321] applied a 3D version of EDSR for MRI brain super resolution. Other approaches proposed for MRI imaging the use of GANs [322]. Finally, the authors of [323] proposed a network for resolution enhancement of 3D MRI brain images named (DCSRN) based on a densely connected structure which outperformed the 3D adaptation of the FSRCNN above mentioned. Concerning 3D CT problems, in [206] a comprehensive literature over the problem is provided for the case of the CT scans of rock samples. The authors compared different literature approaches such as an extended version of adjusted anchored neighbourhood regression algorithm to the 3D case (3DA + algorithm [324], in which a correlative dictionary and mapping matrix between high frequency and low frequency is established to create a 3D HR reconstruction, to the standard bicubic interpolation and the 2D VDSR applied slice-by-slice, and proposed a 3D adaptation of the SRCNN, named 3DSRCNN, which outperformed all the other discussed methods. Other approaches were proposed by [325], where the authors proposed a fully-convolutional network structure composed of two sub neural networks with an intermediate loss to enhance separately the resolution in the two transverse planes and in the longitudinal dimension, obtaining optimal results. Concluding, other interesting approaches for 3D SISR can come from different fields such as [326], in which the authors introduce a particularly lightweight architecture for high quality 3D reconstruction of radar images exploiting a smart revisiting of the convolutions to reduce the number of parameters of the network. Here, we studied and applied 3D SISR to problems of resolution enhancement for tomographic data acquired via TPIFC. As mentioned above, indeed, the lower value of NA characterising the common path setup used for the acquisitions resulted in a sensible loss of resolution for the acquired holograms. Hence, the applications of 3D SISR can be useful to recover it. Here we report the preliminary results

of this study, applying the 3DSRCNN proposed in [300] to our problem of interest.

4.4.1 Dataset creation

To create our dataset, we started from the generation of 1500 model cells of size $128 \times 128 \times 128$ generated according to the realistic cellular model proposed in Chapter 3. The choice of using the cellular model rather than the realistic-looking tomogram model is due to the wish to consider the real RI distribution of the cells, which have internal structures that are both statistically and spatially separated, without the effects of the optical systems influencing the possibility to image this separation. Indeed, the new model was specifically introduced to visually simulate the distortion effects introduced as the light passes through the optical system in terms of loss of resolution. Since here the objective of the network is to recover it, a pristine data is used as a ground truth. Each model cell comprises of 4 mitochondria, a nucleus, a cytoplasm and an external membrane. To obtain the LRE images, the HR ones are first downsampled of a factor 4, then upsampled again using a bicubic interpolation. A yz cut of a pair of LRE and HR tomograms is shown in Fig. 4.15, subfigures (a) and (b). In order to make the training feasible due to memory occupation constraints, the 3D tomograms are cropped into sub-blocks of size 64 partitioning the volume and a "core" sub-block corresponding to the central voxels in a volume of $64 \times 64 \times 64$ voxels. Each element of the dataset undergoes the phase of "network preprocessing", introduced in Chapter 4, to normalise its values, and a data augmentation step, flipping the elements horizontally and vertically. In total, the numerical dataset is composed of 40500 elements. A couple of LRE - HR sub-blocks is shown in Fig. 4.15, subfigures (c) and (d) following the "network preprocessing" stage. Finally, the dataset is partitioned into a training and a validation test sets in proportions of 95:5. Concerning the test set, 50 new model cells were generated and processed as in the case of the training and validation datasets to obtain a test set composed of 450 sub-blocks.

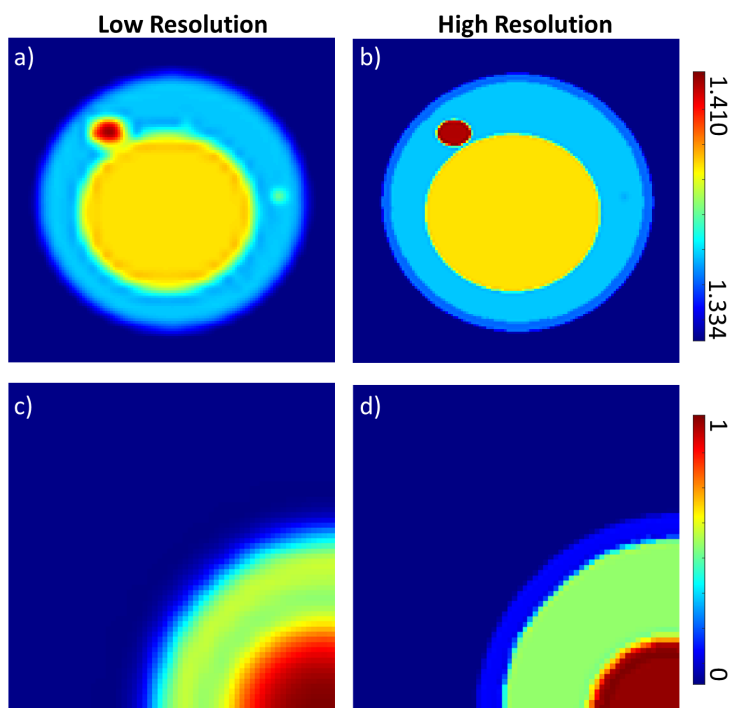


Figure 4.15. Elements of the dataset used for the networks training for the super resolution problem. (a) Central yz slice of a low resolution cell. (b) Central yz slice of the corresponding high resolution cell. (c) Central yz slice of a low resolution sub-block after the "Network preprocessing" stage. (d) Central yz slice of a high resolution sub-block after the "network preprocessing" stage.

4.4.2 3DSRCNN for 3D SR

The network proposed in [300] and shown in Fig. 4.16 is composed of 12 convolutional layers each of them having 64 channels. After each

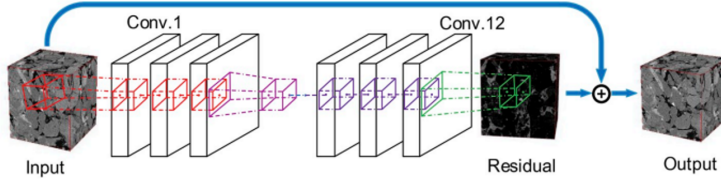


Figure 4.16. Sketch of the 3DSRCNN network. Image reproduced from reference [300].

convolutional layer, the output is zero padded to recover the original size of the input. The first layer extracts a low-frequency patch from LRE images; the middle 10 layers learn the mapping relationship between LRE and HR volumetric blocks, and the last layer combines the learned mapping relation and initial LRE images to finally formulate SR images. After each convolutional layer, a ReLU is used. We exploit an adaptation of this network structure. Due to memory occupation constraints, the number of filters used is 32 for each convolutional layer. As loss we used the MSE as proposed by the authors, optimised using Adam optimiser and using a $LR = 1e - 4$. An early stopping callback is implemented, stopping the training if an alteration over the loss smaller than $1e - 4$ is recorded with a patient of 20 epochs. Moreover, another callback over the value of LR is implemented, lowering it of a factor 10 if a decrement over the loss minor of $1e - 4$ is recorded over 6 epochs. The BS used is $= 16$. Concerning the results of the training, shown in Fig. 4.17 for three samples, they are encouraging. The network successfully learned to counteract the effects of lost resolutions in terms of the spatial regions of RI. As expected, border effects are present and corrupt the reconstruction of the whole volume in the stitching zones between the sub-blocks. This effect might be mitigated in a future version considering overlapping blocks. The Peak Signal to Noise Ratio (PSNR) is used as a metric to quantify the performance of the reconstruction. The values of PSNR mediated over the test set are 39.11 for the 3DSRCNN and 30.10 for the LRE cell. Concerning the application

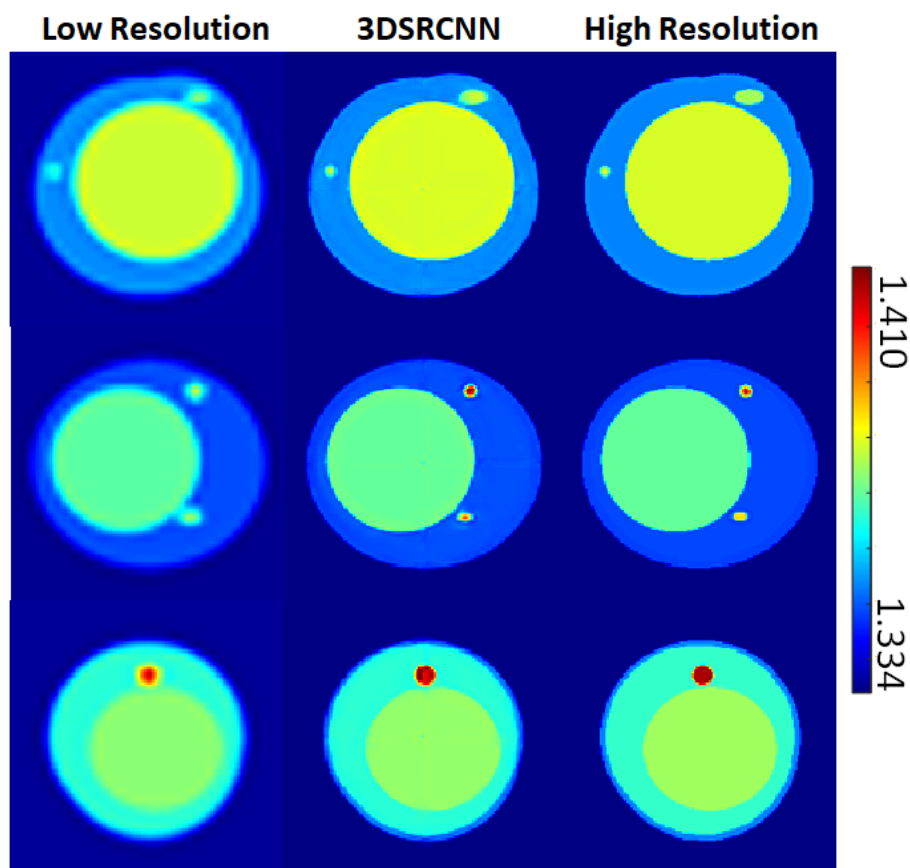


Figure 4.17. Results for 3DSRCNN for three different simulate cells of the test set.

over real data, Fig. 4.18 shows the results for two monocytes from the line THP1. In this case the FBP reconstruction acts as the LRE input. The FBP tomograms of the experimental samples are first reshaped to obtain data of size 128x128x128 and then preprocessed as the simulate data, applying the "network preprocessing" stage and cropping them into sub-blocks of size 64x64x64. The network was able to partly balance the effects of the lost resolution. Here the results are compared to the HOTV2 ones, as it was earlier assessed as a powerful retrieval algorithm for tomographic reconstruction.

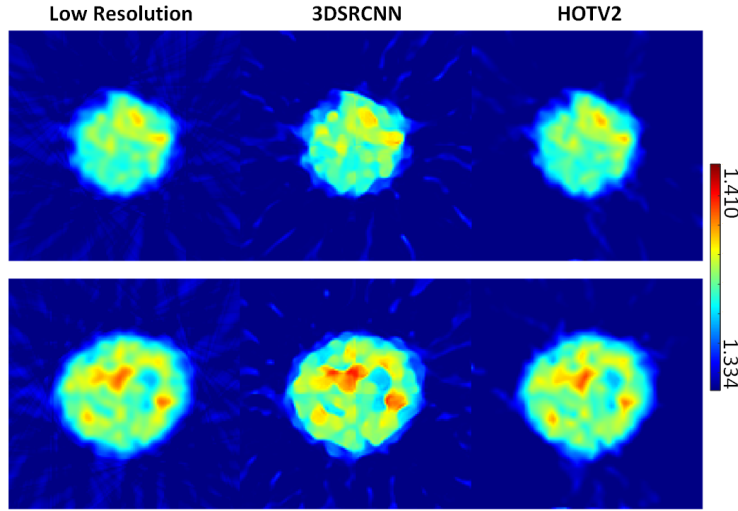


Figure 4.18. Results for 3DSRCNN for two experimental samples (monocytes from line THP1). As a reference, the corresponding HOTV2 reconstruction is reported.

4.4.3 Alternative architectures for 3D SR

As mentioned, for our specific case of interest solutions coming from various field of [SISR](#) can be incorporated. In [326], the network architecture proposed by the authors relies on the consideration that as high-dimensional channel features tend to contain redundancy, reducing the network parameters, which is what makes 3D [SR](#) computation often unbearable, can be achieved through channel compression. Accordingly, they

propose to substitute the direct connection of convolution layers, shown in Fig. 4.19, with the proposed "Fire Block", shown in Fig. 4.19, left. In this image, the two convolution layers architectures are compared, where corresponding colours correspond to analogous dimension. $H_1 \times W_1 \times D \times C_1$ denotes the height, width, depth, and channel numbers of four-dimension tensor, respectively, whereas the notation " $Conv.S_1@1 \times 1 \times 1$ " denotes convolution layer of kernel size 1 and channel number S_1 . Both connections achieve to transform an input of size $H_1 \times W_1 \times D \times C_1$ in an output of size $H_1 \times W_1 \times D \times (E_1 + E_2)$ by a series of convolution layers. The standard convolutional layer achieves it exploiting one convolution layer with kernel size 3. Conversely, the 'Fire' module comprises of two stages: the 'Squeeze' stage and the 'Expand' stage. In the 'Squeeze' stage, the feature of size $H_1 \times W_1 \times D \times C_1$ is passed into one convolution layer with kernel size 1 to obtain the feature of size $H_1 \times W_1 \times D \times 4 \times S_1$. Then, this feature is fed into two different convolution layer branches with kernel size 1 and 3 to obtain two features of size $H_1 \times W_1 \times D \times E_1$ and $H_1 \times W_1 \times D \times E_2$, respectively. Finally, these two outputs are concatenated in the channel dimension by the 'Expand' stage to reach the output dimension of $H_1 \times W_1 \times D \times (E_1 + E_2)$. As estimated by the authors, the number of local networks parameters can be reduced to 1/4 of the original number by exploiting the fire blocks instead of standard convolutional ones due to the use of 1-size convolutional kernels instead of $3 \times 3 \times 3$ ones. The whole network structure of SRCNN is constructed as in figure 4.20, where in a standard UNET architecture the convolutional blocks have been replaced by the fire modules. In our case, the input consists in $128 \times 128 \times 128 \times 2$ data. Note that, given the particularly lightweight nature of the CNN, it was possible to train the network without cropping the input data into sub-blocks as for the previous case. The stride size of max pooling layer is 2 except for the bottom layer of the UNET where it is set = 4. The sizes of corresponding transpose convolution layers are reversed. In our case, $E_1 = E_2 = E = 3$ in the first layer, while $S_1 = C_1 = 2$. The second colour channel, which for the authors corresponded to the imaginary part of their input, in our case was set to a zero-valued matrix given the real-valued nature of the RI. The network was trained building a training, and a validation and a test set with the same sizes as for 3DSRCNN. The chosen loss is the MSE, optimised via the Adam optimiser using a $LR = 1e - 6$ exploiting a callback meant to reduce

its value of a factor ten if a variation over the 0.0001 with a patience of 6 epochs. A similar callback is implemented over the training epochs, where a patience of 20 was used. The BS is = 4. The total number of parameters of the network is 15567.

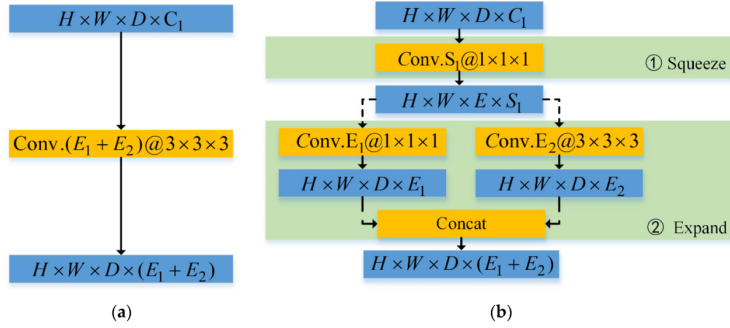


Figure 4.19. (a) Conventional convolutional layer. (b) "Fire module" proposed in [326]. Image reproduced from [326].

Concerning the results, however, the network did not produce satisfying outcomes, even in the described best combination chosen for the hyper-parameters. Fig. 4.21 shows an example of the network output for three elements of the test set. As visible, the network is not able to recover the lost resolution and to re-establish the correct shape of the internal structures and membranes. The value of PSNR mediated for all the elements of the test set reconstructed by the network with respect to the HR ground truth is equal to 31.91, whereas the same value computed between the input LRE images versus the HR ground truth is equal to 29.71, underlying the minimal improvement brought by the network use.

4.5 AI to identify tumour cells

Nowadays, the gold standard technique for cancer diagnosis is tissue biopsy. This investigation method, even though highly reliable, shows some important drawbacks. First, the examination is highly invasive and uncomfortable for the patient. In addition, it cannot be repeated frequently over the same body region and the istological examination to produce results requires a long time. Generally, one of the major concerns regarding cancer

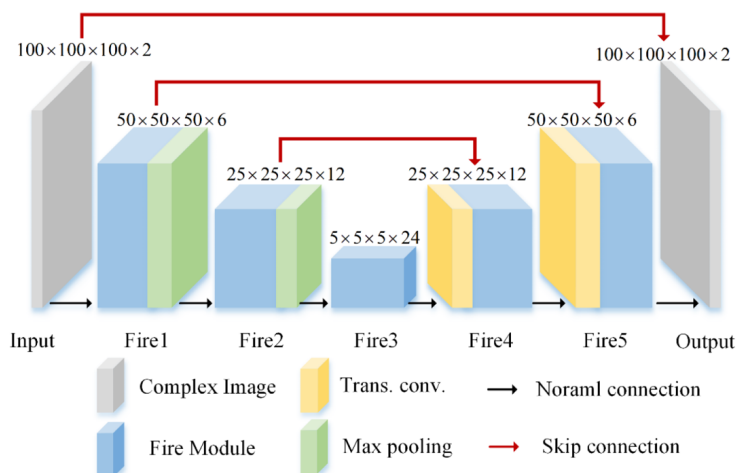


Figure 4.20. Architecture of the "Fire UNET" proposed in [326]. Image reproduced from [326].

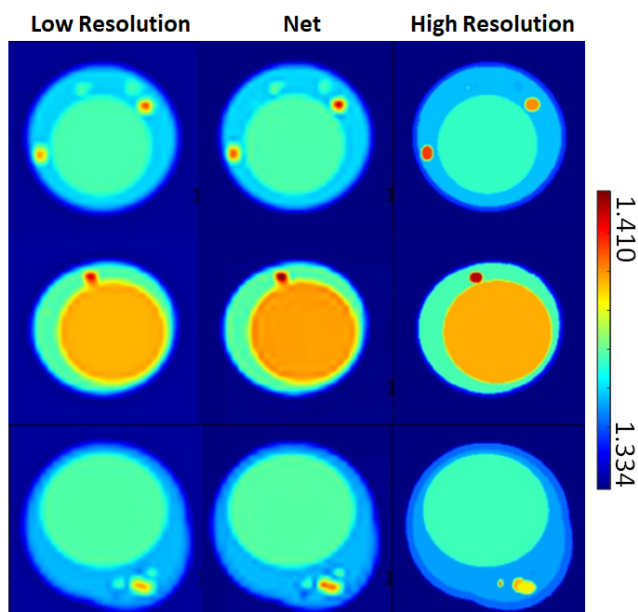


Figure 4.21. Results for 3DSISR performed using the "Fire Unet" architecture proposed in [326] for three elements of the test set.

detection nowadays is related to the early-diagnostics possibilities, which are linked to the sensitivity of the identification exam carried out. Often, classic screening techniques such as CT, MRI and Positron Emission Tomography (PET) require the suspect mass to reach a determined dimension to be compatible with the sensitivity of the examination and more generally to be detectable by tissue biopsy. As a consequence, once that the physical inspection is carried out, the mass has already reached considerable dimension and might represent a concern for the patient's health. One of the possibilities which is highly under medical research attention is the one to look for material present in the bloodstream which is unequivocally linked to the presence of the tumour. This new diagnostic technique, called LB, is quickly becoming a valuable diagnostic tool often paired to the standard ones. This perspective is interesting as it was demonstrated that biological masses release second-order biological material before they reach the sensitivity of standard diagnostic techniques [209]; therefore, the identification of this material is due to the presence of the tumour in the body. The major strengths of this approach are linked to its small intrusiveness, the short time needed to perform it, the possibility to repeat it frequently and the possibility it gives to produce immediate feedback for the validity of the therapy performed, as the evolution of the detected material might help to tailor the treatment to the patient's condition. In particular, an interesting possibility is to detect the so-called CTCs, which are cells released from the primary or the metastatic tumour which enter the bloodstream. Nowadays, the search for CTCs is made mainly looking for a specific indicator which unequivocally distinguishes the CTCs among all the material in the bloodstream. Many choices are possible and extensive research is made over this topic. The most used indicators are cancer-specific surface antigens [174] and [327, 328, 329, 330, 331, 332, 333, 334, 335]. This method, even though highly used in diagnostics, often leads to problems of false negative results, given the great heterogeneity of tumours. Consequently, different techniques have been developed which rely on the recognition of peculiar functional and metabolic properties of the CTCs, such as Matrigel inversion capacity [336], glycolic metabolism [337]. However, the drawback of the use of these indicators is related to the low throughput they allow which is not compatible with cancer screening programs. Techniques such as next generation sequencing allow to identify tumour-specific mutations

in the bloodstream, however, they often require technological complicated processes to be carried out over CTCs, which first have to be identified and isolated and then chemically processed, determining their destruction. On top of all the mentioned, the identification process becomes a lot more complicated as the CTCs are incredibly rare in the bloodstream, with 1-10 occurring for 1 mL of blood [338]. The major drawback of the presented techniques lies however in the fact that the indicator must be known a-priori. This peculiarity represents the biggest limitation of LB, as given the great inter-variability of tumour cells the indicators are often unavailable, determining a serious limitation for the high potentialities of the LB. Alternatively, promising solutions can be found resorting to IFC, and in particular label-free IFC techniques such as DH, as the QPMs encode metrological properties of the sample that can be useful in the discrimination of different populations. Here, the possibility to conjugate DH-IFC with AI techniques is discussed for the problem of discrimination between CTCs and healthy blood cells. In particular, the binary classification between model CTCs and monocytes is performed. The choice to discriminate only between CTCs and monocytes is due to the fact that it is easy to eliminate other components of the bloodstream by physical filtering techniques due to their different sizes, conversely, monocytes exhibit a morphology really close to CTCs. Two different classifications techniques will be analysed, in particular a classification based on a ML approach, and one based on a DL approach. In the analysis of the performance of different classifiers for this application, primary concern must be put into the choice of a method of great accuracy which lowers as much as possible the number of false negatives. This is linked to the extremely rarity of CTCs in the bloodstream; as a consequence, a low value of accuracy would mean to lose the precious information a single detected CTCs carries. In addition, considering the high clinical significance of a false negative result, which corresponds to the lack of a tumour identification, this parameter has to be minimised as much as possible.

4.5.1 Dataset creation

The dataset in use was composed of 2550 model Ovarian Cancer (OC) cells from the line A2780 and 401 model THP-1 monocytes. The holograms of the cells were acquired in an in-flow DH conditions with the setup pre-

sented in Chapter 3 and processed with the pipeline described in Chapter 2 to obtain a total of 2951 QPMs. The dataset was relatively small and strongly unbalanced. This was a consequence of working with experimental data, which resulted in having a ratio of 1 monocyte for each 6.36 OC cells, adding challenges to the classification problem. However, in this case the dataset was unbalanced in a good direction for our problem, according to the considerations made above. Since the need is to be able to classify with the lowest false negative number as possible, it is beneficial to train the classifiers exposing them to the highest number of CTCs as possible. The dataset was split in training set (97.3%) and a test set (2.7%), with a holdout partition for the ML classification. The test set was chosen in a balanced fashion, containing the same number of CTCs and monocytes.

4.5.2 ML approach

4.5.2.1 Feature selection

All the QPMs available were processed to extract 12 features of common interest belonging to three different categories: morphological features (area expressed in pixels, eccentricity, perimeter expressed in pixels), texture-based features (homogeneity, energy, kurtosis, skewness and entropy) and QPI-related features (maximum, minimum, mean and standard variation of the QPM). The morphological features were extracted considering a binarized version of the QPMs obtained with a thresholding technique, whereas the texture-based features were obtained using a grey level co-occurrence matrix, which expresses how combinations of discretized values (gray levels) of neighbouring pixels are distributed along one of the image directions. The QPI features were extracted directly from the QPMs and, since the latter are measurements of the optical path delay in the cell, they carry information about the cell's RI. It is worth pointing out that the QPI features would not be available with a conventional imaging technique. The first analysis conducted was a feature study and selection over the ones available. To begin, both a PCA and T-SNE [339] analyses were conducted over the entire pool of cells to study their clustering properties. As cleared by Fig. 4.22, the samples do not tend to naturally cluster into two classes according to the features available. The classification problem is, therefore, non-trivial and must be addressed

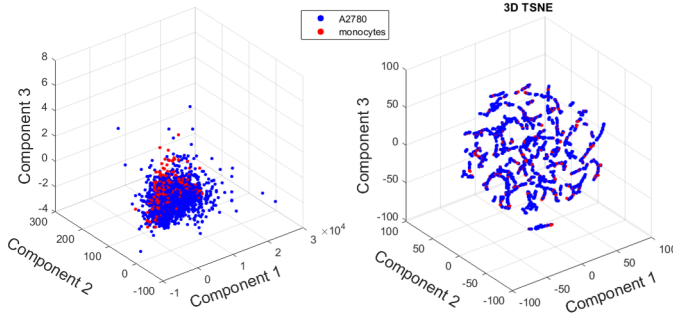


Figure 4.22. PCA and T-SNE carried out over the data before feature selection to assess the complexity of the classification task by observing the natural data clustering. Image reproduced from reference [340].

with AI aided techniques. Based on the work proposed by [341] a study was conducted over the features to understand their level of significance. The Kendall correlation coefficient was evaluated over all the features, after proving that due to their low numerosity, the data did not follow a normal distribution. According to the values of the Kendall correlation coefficients, shown in absolute value in Fig. 4.23, the morphological and the texture features do show some correlation with statistical relevance ($p\text{-value} < 0.05$). Differently, the QPI features (highlighted by the red box in Fig. 4.23) show a low intra-correlation value of and a very low correlation with the other features; both these observations are of statistical relevance ($p\text{-value} < 0.05$). Accordingly, we can deduce that all the QPI features carry individual information of statistical relevance from the classification point of view. Then, excluding non-statistically relevant values of correlation coefficients ($p\text{-value} > 0.05$), a threshold $R = 0.9$ was set to determine which features were mutually correlated. The only two features falling into this category are the kurtosis and the skewness; therefore, the kurtosis feature was discarded as redundant. Then, we applied the Relief Algorithm [342] to quantitatively assess the level of informativeness of the features available. The results are shown in Fig. 4.24, with the red arrow pointing out at the QPI features. As expected, they occupy high level of ranking, therefore confirming their importance in the classification task. Concluding, we again performed a PCA and T-SNE analysis and,

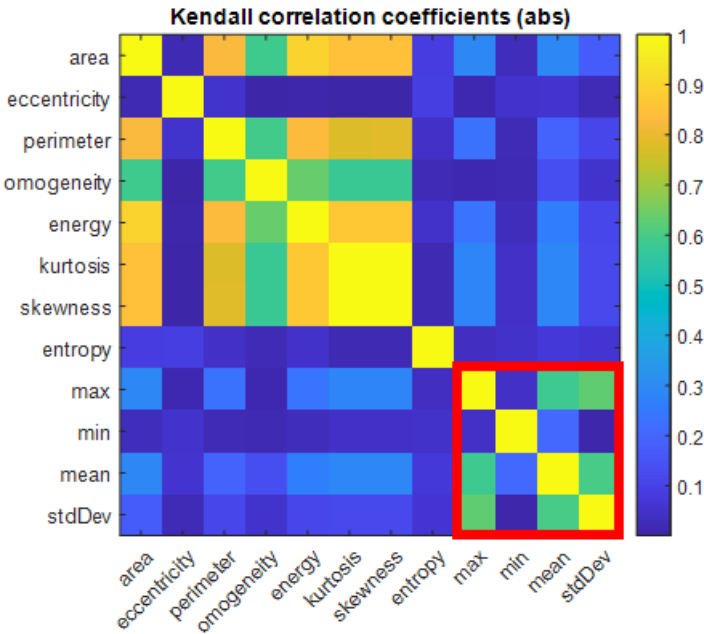


Figure 4.23. absolute value of Kendall correlation coefficient among the inspected features. The red square marks out the QPIs features. Image reproduced from reference [340].

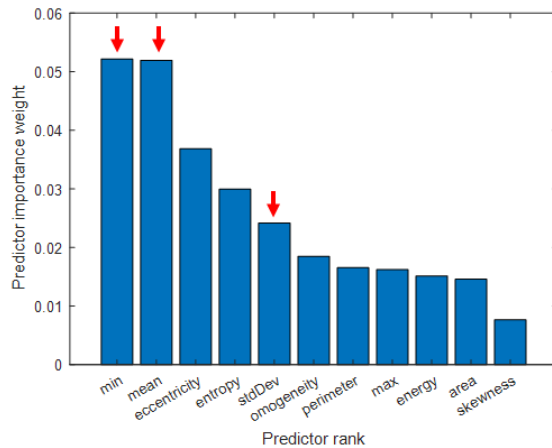


Figure 4.24. Results of Relief analysis performed over the features to assess their significance. The red arrows point out some of the QPIs features which are among the most informative. Image reproduced from reference [340].

as expected from the feature selection process which assessed that all the features available were informative and non-redundant, no improvement was obtained in the clustering of the samples, as shown in Fig. 4.25.

4.5.2.2 ML classification results

The classification task was performed using the MATLAB Classification Learner framework. Different standard classifiers were trained making use of a cross validation with a 10-class K folding implemented over the training set described above to improve the robustness of the analysis. The classifiers were tested in different condition. First, all the features available were used. Then, we selectively excluded selected groups of features among the three groups available (morphological, texture, QPI) while keeping all the others to assess the informativeness they add for the classification. Table 4.8 reports the results for the validation and test accuracy obtained in the case-studies. Column 1 is relative to the case for which all the features were used, while columns 2, 3 and 4 are relative to the cases in which the morphological, texture and QPI features were respectively excluded while including all the others. The case in which all the features are used

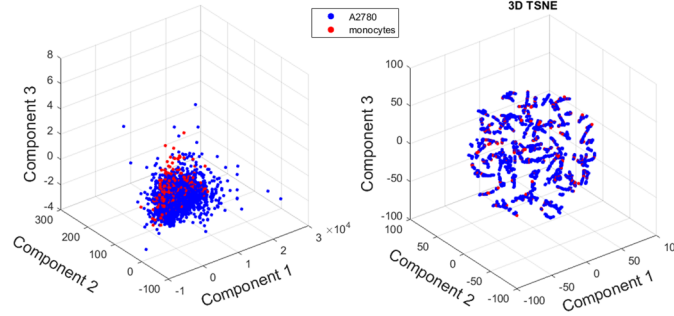


Figure 4.25. Results of PCA and T-SNE analysis after the feature selection process. Image reproduced from reference [340].

Classifier	All Features		No Morph.		No Texture		No QPI	
	Val.	Test	Val.	Test	Val.	Test	Val.	Test
Fine Tree	93.3	85.0	93.9	83.8	90.8	81.2	89.3	75.0
Med. Tree	93.8	81.2	93.7	80.0	92.2	73.8	90.3	67.5
Coarse Tree	92.2	72.5	92.2	72.5	90.1	63.7	90.8	62.5
Linear								
Disc.	93.3	77.5	92.4	75.0	92.5	76.2	90.5	67.5
Quadratic								
Disc.	94.7	88.8	93.2	78.8	93.5	82.5	90.5	76.2
Linear SVM	93.9	80.0	93.8	80.0	92.6	78.8	91.4	66.2
Quadratic SVM	96.7	88.8	96.6	87.5	94.8	87.5	92.3	72.5
Cubic SVM	96.8	90.0	96.1	87.5	95.3	85.0	93.3	80.0

Table 4.8. Validation and test accuracy percentage evaluated for different classifiers considering different features groups.

presents the highest values of validation and test accuracy. This is compatible with the results found studying the features available, where it was assessed that all the features carried valuable information for the training. The biggest drop in the accuracy was when the QPM features were eliminated, confirming that the physical knowledge that a quantitative approach such as DH carries into the acquired images is greatly beneficial for the applications and justifies in full the use of a QPI method for coping with this classification issue. However, in general, the values of accuracy were very high with a significative decrease in the test accuracy, symptom that some overfitting over an insufficient dataset might be present for a ML classification task. The validation and test confusion matrix relative to the Cubic SVM classifier are shown in Fig. 4.26 for the case of all the features are employed. Generally, this is the classifier which in average shows the best performance (highlighted in red in Table 4.8). As indicated by the

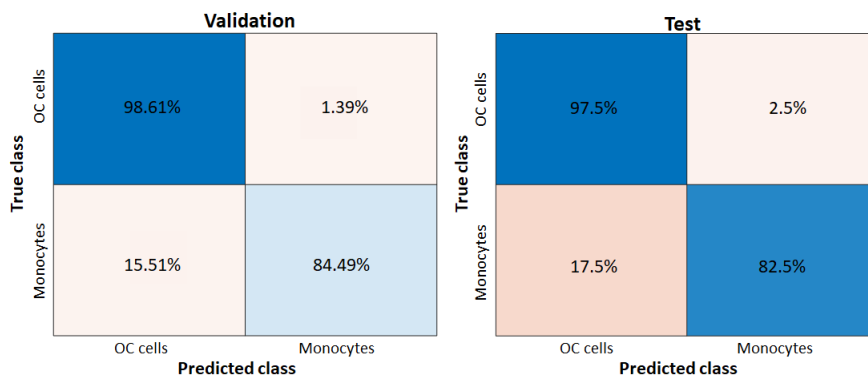


Figure 4.26. Validation and test confusion matrices relative to the Cubic SVM classifier when all the features are employed. Image reproduced from reference [340].

test confusion matrix, there is a high number of monocytes incorrectly classified as OC cells (17.5%); conversely, just 2.5 % of the model CTCs is incorrectly classified as monocytes and, therefore, missed from a point of view of detection. However, given the high rarity of CTCs in the blood-stream compared to monocytes, such a high percentage of misclassification might lead to a complete loss of the CTCs. Concluding, the non-optimal classification results obtained via ML are coherent with the almost absent natural clustering of the data, symptom that a ML approach might not be the most effective to handle the problem complexity with the type of experimental dataset available.

4.5.3 Deep learning classification

DL approaches are often greedy in terms of number of images composing the dataset. Therefore, given the reduced cardinality of the one available, we employed standard CNN architectures pre-trained over the ImageNet [343] dataset. The possibility of using a transfer learning approach again highlights the power of including image-based data driven approaches into LB methods, as there is the possibility to benefit of a huge literature regarding image processing and Computer Vision applications. Four different network architectures were used for the discussed task:

MobileNet V2 [344], MobileNet V3 small, MobileNet V3 large [345] and ResNet-18 [346]. ResNet-18, in particular, is an interesting network architecture which solves the problems of vanishing and divergent gradients by introducing skip connections. This problem is linked to the exponential diminishing of the gradients during the backpropagation stage and is due to the repeated application of activation functions with derivatives that tend to be small, like sigmoids or hyperbolic tangent. The vanishing gradient problem often obstructs information transmission from the shallow to the deep layers of DCNNs, limiting their ability to learn complex representations and thus lowering their diagnostic performance. Introducing the skip connections makes the training of deep networks viable, as the gradients can directly flow through building blocks and the vanishing gradient problem can be avoided; hence making the training of the network easier. ResNet-18 therefore is able to overcome the problem and to provide great generalisation performance with a relatively small number of parameters. Indeed, ResNet has been successfully used for many medical image classification tasks [347]. The architecture of the original ResNet-18 is shown in Fig. 4.27 (b), composed of 17 convolutional layers, one fully connected layer and a SoftMax layer for the classification for a total of 18 layers.

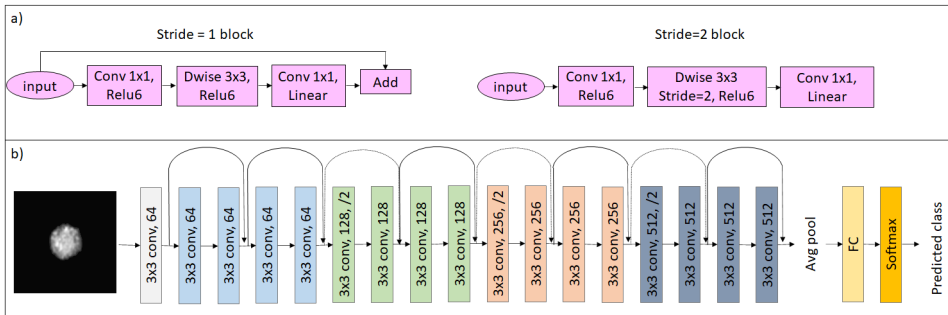


Figure 4.27. Subfigure (a) Detail of the Mobilenet V2 architecture and subfigure (b) ResNet-18 architectures used. Image reproduced from reference [340].

The convolutional kernel size is 3x3. The network is designed in such a way that if the output feature map is the same size, then the layers have the same number of filters. However, filters get doubled in layers if the output feature map is halved. The downsampling is performed using

Network Architecture	Validation Accuracy	Test Accuracy
MobileNet V2	95.2	93.2
MobileNet V3 Small	79.2	79.7
MobileNet V3 Large	88.6	87.9
ResNet-18	94.0	94.0

Table 4.9. Results summary of the validation and test accuracy over all folds.

convolutional layers with stride of 2. Two types of residual connections are present in the network. The first type, denoted by solid lines, is used when input and output have the same dimensions. The second type, denoted by dotted lines, is used when dimensions increase and still performs identity mapping but with zero padding for increased dimensions with a stride of 2 [348]. Concerning the MobileNet V2, its basic block is the inverted residual bottleneck shown in Fig. 4.27 (b). This block is composed of a 1x1 pointwise convolution layer expanding the input into a higher dimensional space followed by a 3x3 depth-wise convolution. Finally, a second pointwise convolution reduces to a smaller dimension and residually connects to the input layer. All convolutional layers are followed by a ReLU activation function. The MobileNet V3 inverted residual bottleneck is similar to the one of the V2 with the addition of the squeeze-and-excite block [349], which executes average pooling to a vector composed of two fully connected layers and multiplies the produced vector with the original matrix. In this architecture, instead of the ReLU the hard-swish activation function is used. MobileNet V3 small and MobileNet V3 large differ by the amount of inverted residual blocks and of number of channels in each block, 11 and 15 respectively. The input to the nets for the training are the QPMs without any further processing. The training was performed over the training set with a five-fold cross validation. For each fold, the network was trained for 10 epochs with a batch size of 16, using the ADAM optimiser [350], the cross entropy loss function, and a learning rate of $1e - 4$.

The results for the classification task are reported in Table 4.9, showing the validation and test accuracy over all folds for each network architecture. In general, the values of accuracy are high, specifically in comparison to the conventional ML approach. The networks produce similar results, with a slight improvement using the ResNet and the Mobilenet V2. Fig. 4.28

reports the validation and test confusion matrix relative to MobileNet-V2 classifier. Even in this case, in both validation and test confusion matrices there is an elevated number of monocytes misclassified as OC cells (5% and 7.5%, respectively), however, no model OC cells unidentified. This agrees with the fact that the population of the cells were not balanced. The 0-value of false negative events is particularly important and sought after, as detailed above, therefore suggesting more promising results for the DL classification approaches compared to the ML ones.

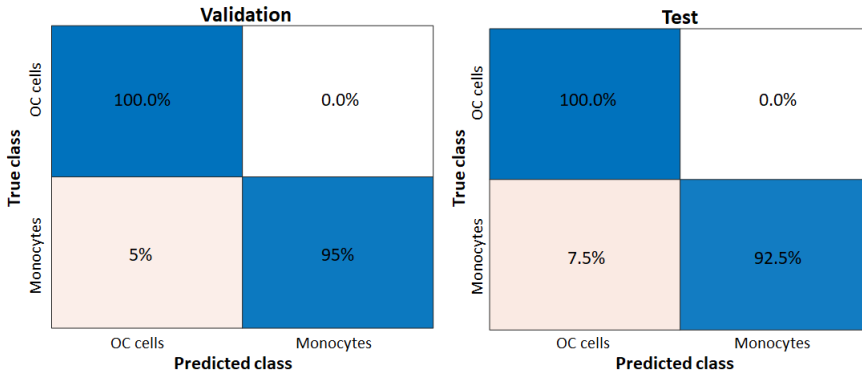


Figure 4.28. Validation and test confusion matrices relative to MobileNet-V2 classifier. Image reproduced from reference [340].

4.6 DL for cells classification based on multiple holographic projections

The objective of this study was to again incorporate AI techniques in conjunction to DH for diagnostic purposes in the framework of LB. This time, DL was used to classify between benign and malignant epithelial mammary cells and between four different types of WBCs, including activated ones. Concerning the methodological point of view, as discussed above, incorporate DH into classification tasks allows to obtain a great additional information which is beneficial from the classification point of view. Clearly, fully exploiting the 3D information encapsulated by DH would constitute a further important improvement in terms of informa-

tion added and several papers have discussed this approach [37, 351, 263]. However, the tomographic reconstruction is a heavy task, as first the projection angles in an in-flow conditions have to be identified and later the reconstruction performed. Therefore, it is a time and resource consuming stage which is generally not compatible with real time analysis. Here, we propose to encapsulate differently the 3D information, without resorting to the full tomogram reconstruction. We feed the network with QPM corresponding to different information keeping trace of the angular variation but without the need to estimate the angles completely, sparing a lot of computational resources. Finally, the integration of DL allows for automated and rapid analysis of large volumes of data, facilitating high-throughput cell classification. IFC suffers from low throughput as the need to image clear and focused image is necessary. The proposed technique can be used for increasing the classification accuracy or decreasing the number of recorded cells needed for training while saving the collection of training examples. The discussed model has great potentiality concerning various medical applications. For example, concerning the identification and characterisation of CTCs in LB applications, which are very rare in the blood, by recording several projections of them rather than one, a better exploiting of this rare information can be obtained, thus enabling finer detection or monitoring of disease progression [340, 352]. In regenerative medicine, our method can facilitate sorting and enrichment of unstained stem cells, to be further used for tissue engineering and cell-based therapies [353]. Concerning the classification of WBCs, we exploited T-cells in three states to compare similar cells as a difficult classification task. This application can be useful for the identification of T-cells in different stages of activation, which has high importance in detecting inflammation and sepsis [354, 355].

4.6.1 Holographic dataset creation

4.6.1.1 Sample preparation and acquisition

The first experiment was conducted over model cell lines of metastatic breast carcinoma cells MCF-7 and normal human mammary epithelial cells MCF-10A. The samples were prepared as discussed in [225] and acquired with the setup described in Chapter 3. At the end of the experiment, a total of 1521 cells were acquired: 555 MCF-7 and 966 MCF-10A. The

second experiment was conducted on real **WBCs** in a study approved by Tel Aviv University's IRB. Blood was acquired from the Israeli National Blood Services and was used to isolate two types of **WBCs** with a procedure described in [356]: Neutrophils and T-cells, with the latter undergoing also a procedure to activate them and consequently incubated for 3 and 7 days. Therefore, in total four types of **WBCs** were available: Neutrophils, inactivated T-cells, activated T-cells with 3 days incubation and activated T-cells with 7 days of incubation. The digital holograms of the **WBCs** were acquired in an in-flow modality via an off-axis interferometric microscope in a Mach-Zehnder configuration realised at Tel Aviv University and depicted in Fig. 4.29 [356], characterised by a reduced **NA** of 0.66. The setup works in a slow flow of $5\text{--}10\ \mu\text{l}$ per hour, ensuring that the cells will roll on the bottom of the channel and therefore eliminating the focusing problems as all the cells are on the same plane. In total, 2997 **WBCs** were acquired, split into 933 neutrophils, 877 T-cells, 401 activated T-cells, 3 days old, and 786 activated T-cells, 7 days old.

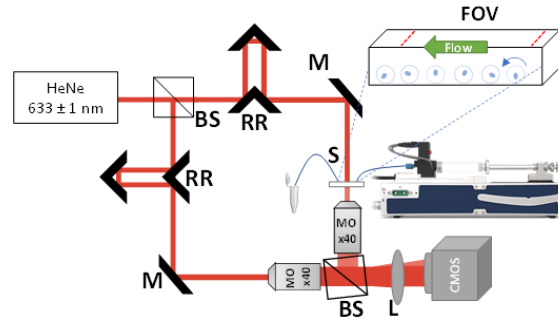


Figure 4.29. The optical system used for the **WBC** experiment, a Mach-Zehnder microscope interferometer. BS, beam splitter; RR, retroreflector; S, sample; MO, microscope objective; M, mirrors; L, tube lens; CMOS, digital camera. Cell flowing in the micro-channel was induced by a syringe pump. Image reproduced from reference [356].

4.6.1.2 QPMs processing

Concerning the processing of the **WBCs**, the recorded interferogram videos were cut into 100 frames and processed with a numerical pipeline

comprehending the same steps as the one described in Chapter 2. To compensate for noise and aberrations in the holograms a reference hologram was used, obtained for each 100-frames batch averaging the acquired QPMs, given their sparsity in the FOV and their constant flow. The unwrapping step is made according to [357]. Then, each frame went through a segmentation process comprising different steps: first, the frame was binarised using a threshold of 0.02 micron, then a single cell is identified in the FOV by tracking the centroid of the specimen of interest and finally, following the position of the centroid of the cell of interest in the different frames, a multiplicity of QPMs corresponding to one cell was obtained. At the end, the cell images were zero-padded to have the size of 104×104 pixels and saved as 24-bit colour images using the JET colormap. An example of QPMs is shown in Fig. 4.30.

4.6.2 Neural network architecture

For the classification task, the same ResNet-18 as in the previously discussed case was used as the backbone for the CNN here employed. The choice of use of the Resnet-18 is due to the good handling it offers of the vanishing gradient problem while still efficiently solving the classification task. Concerning the input to the network, stacking all the available projections of a single cell over the colour channel of the data would only provide sub-optimal outcomes, given the non-rotational invariance of the 2D filters. In fact, this approach would assume that the observed cell possesses the exact same viewing angle across all input images within the channel, which is non-true given their flow and roll into the channel. To address this limitation, a more effective strategy was employed. This strategy accounts for the different viewing angles of the cell which are available and, if provided to the network as separate input, consists in an additional source of information available for the cells. This ensures a more comprehensive representation of the cell appearance from various perspectives, acting as experimentally-derived data augmentation via the 3D rotation and allowing the network to learn and differentiate the features associated with different viewing angles. To effectively incorporate the information regarding the multiple views, we resorted to the late fusion approach [358]. Accordingly, our network has multiple heads (indicated by the red dashed box in Fig. 4.31), each of them built from ResNet-18 backbone described

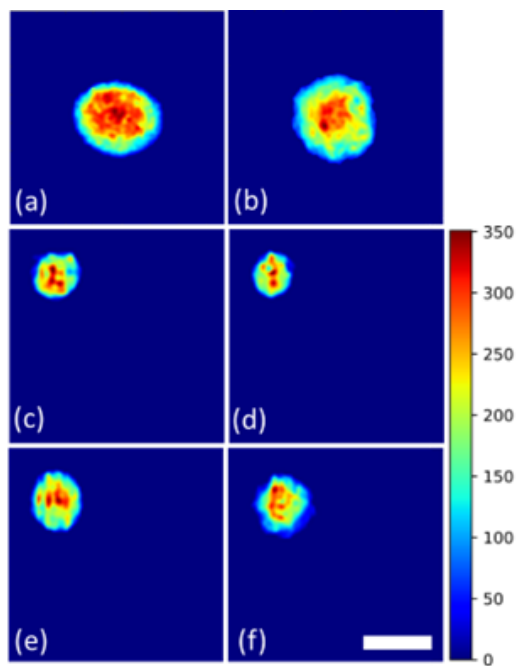


Figure 4.30. Examples from the acquired database of quantitative OPD images of cells. Color bar represents OPD values in nm. First experiments: (a) MCF-7, (b) MCF-10A. Second experiment: (c) Neutrophil, (d) T-cell, (e) Activated T-cell (3 days old), (f) 7-day activated T-cell. White bar indicated 10 μm . Image reproduced from reference [356].

above in Fig. 4.27 (a). Each head receives as input one viewing-angle projection, as different view-angles projections contain different information. The final latent vectors are concatenated together and are fed to a fully connected layer and a SoftMax classification function. For each discussed experiment, we built a network with a number of heads corresponding to the projection used and two or four output classes depending on the classification task of interest. All the DL infrastructures were based on PyTorch v1.13.1 + cu11.7, with architectures taken from TorchVision v0.14.0 + cu11.7 uploaded with pre-trained ImageNet weights.

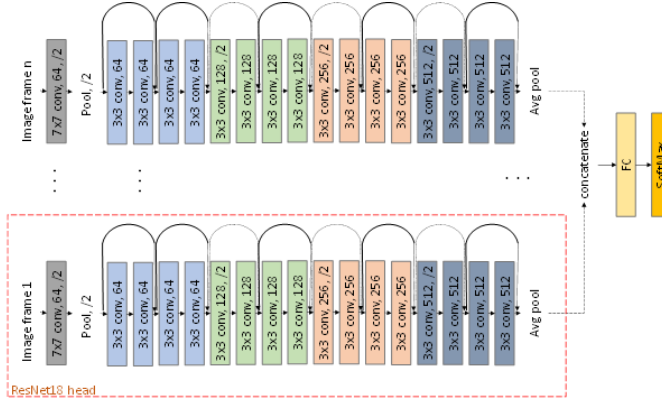


Figure 4.31. Sketch of the ResNet-18 architecture used for the classification task. Image reproduced from reference [356].

4.6.3 Performance evaluation

4.6.3.1 First experiment: Breast-cell-lines classification

In this case, the objective of the classification task was to discriminate between cancer and non-cancer breast cells. Concerning the total of 1521 breast cells acquired, composed of 555 MCF-7 and 966 MCF-10A, for each cell five different projections were available. The dataset was split into a training set (60%), a validation set (20%) and a test set (20%). The network was trained with a $BS = 64$ and using the Adam optimiser [350] to minimise a cross-entropy loss. The LR was fixed at 0.0001 for the first epoch and linearly decreased to zero over the next 30 epochs. The training

was repeated using from just one projection up to the maximum number available equal to five. Fig. 4.32 reports the validation and test accuracy as a function of number of cell projections used for the training. Since this first classification task was relatively simple, the accuracy values were high even using only one projection, achieving an accuracy of 96.4% on the validation set and 94.8% on the test set. However, increasing the number of projections per cell strongly improved the accuracy values, up to to 99.7% in training and 98.7% in the validation. Fig. 4.33 reports the confusion matrices of the experiments for the cases 1 and 5 projections used. In both confusion matrices, there is an elevated number of MCF-7 incorrectly classified as MCF-10A (3.09% and 2.06%, respectively). This agrees with the fact that the two populations of cells were not completely balanced. However, the use of 5 projections has a great impact of the number of MCF-10A incorrectly classified as MCF-7, which is decreased from 9.01 % up to 0. This is an important result, since as discussed the minimisation of false negatives is pivotal when looking for rare CTCs in diagnostic-linked applications such as LB.

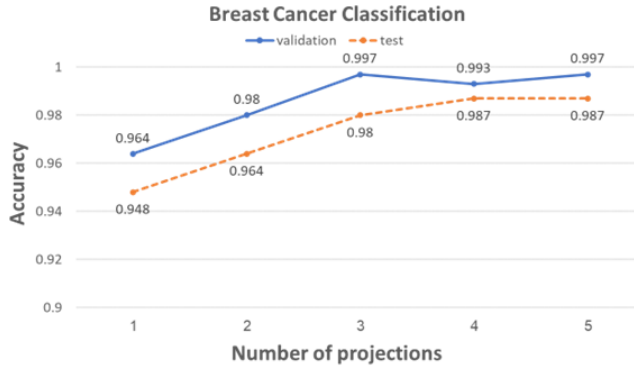


Figure 4.32. Results for the first classification task: validation and test accuracy as a function of number of cell projections used for the training. Image reproduced from reference [356].

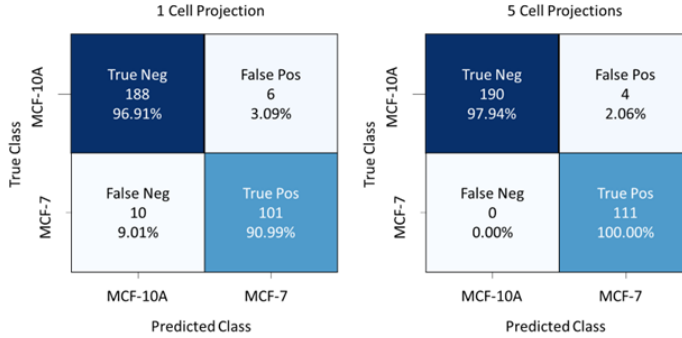


Figure 4.33. Confusion matrices for classification between healthy and cancer breast cell lines (MCF-10A and MCF-7), comparing one and five cell projections. Image reproduced from reference [356].

4.6.3.2 Second experiment: WBCs classification

The second classification task performed was a more challenging one, where the network was trained to discriminate among four similar types of WBCs. The dataset is composed of 2997 WBCs made of 933 neutrophils, 877 T-cells, 401 activated T-cells, 3 days old, and 786 activated T-cells, 7 days old. For each cell 12 different projections were available. The dataset was split between a training set (75.6%), a validation set (15%) and a test set (9.4%). The network was trained with the same settings as for the breast cell lines. The LR was kept constant at 0.0002 for the first 25 epochs and linearly decreased to zero over the next 25 epochs. For this classification problem, three different scenarios have been studied: (i) we fixed the training set size to the entire training set of 2265 cells and, as for the previously discussed classification task, we repeated the training increasing the number of projections used from 1 to 12. The results are shown in Fig. 4.34 (a), blue line. We achieved classification accuracy ranging from 88.6% when using one projection per cell, up to 93.8% when using 12 projections per cell. (ii) we increased the number of projections used and decreased the dimension of training set accordingly, so that each experiment had 2265 input images in total. The results are shown in Fig. 4.34 (a), (b) with the orange dashed line. Our results show that the classification accuracy remains approximately constant at 89% despite

the decrease in training set size. (iii) we fixed the number of projections to one, as in a conventional classification case, and tested the accuracy measure as a function of the decreasing training set size. The results are reported in Fig. 4.34(b) with the grey dot-dashed line. The maximum achieved classification accuracy is 88% in the case of the largest dataset and decreases down to 80.8% in the case of the smallest dataset, as would be expected. Concluding, the results show a great improvement in the performance if the number of projections used is incremented, whereas stationary results are obtained when the training set size is decreased and the number of projection included increased. This observed stability in accuracy suggests that the model can maintain its performance even with a smaller training set if a sufficient number of projection is included. Indeed, even when decreasing the training set size significantly to 8% using only 188 cells, just by using more projections, the accuracy can be improved by almost 10%. From both tests, we can understand that as the classification task is more complex, as in the second experiment of similar WBCs, more projections are needed, but we could quickly achieve a flatten accuracy curve for the number of projections. The comparison in the third scenario with conventional classification approach demonstrates the improvement brought by the use of multiple projections respect to using only one, which is more prominent as the dataset size decreases. This highlights the critical importance of informative training examples in order to achieve optimal performance in classification tasks involving limited data.

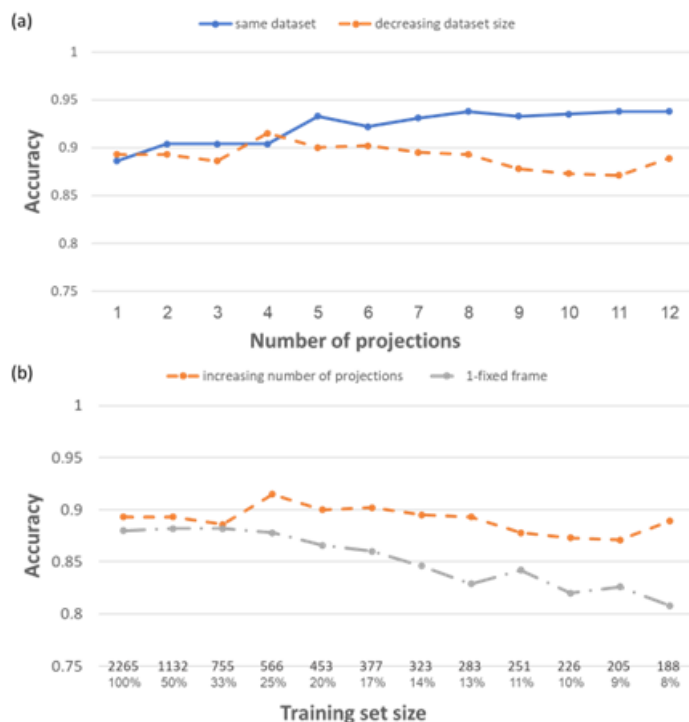


Figure 4.34. Accuracy curves comparing three different scenarios. (a) In solid blue, the first scenario of classification: using the full training set with increasing number of projections per cell. In orange dashed, the second scenario: increasing the number of projections number while decreasing the training set size. (b) In gray dashed dotted, the third scenario: using only one projection while decreasing the training set size (the x-axis shows the set size and percentage), in comparison to the 2nd scenario. Image reproduced from reference [356].

Second order Rytov modelling of the forward scattering

The mathematics of inverse scattering problems is important in many disciplines such as optical imaging, microscopy, radar and medical imaging [359] and constitutes the base of the tomographic reconstruction. The term forward scattering is used to describe the direct problem, in which the scattered field is estimated starting from a known distribution of RI, whereas the term inversion describes the procedure of estimating the original RI distribution starting from the profile of scattered field. Clearly, the two descriptions are intimately linked, as a correct modelling of the forward problem influences the inversion results. The relationship between the scattered field and the originating distribution is extremely complex and often non linear. To approach it, approximations are frequently introduced, such as the well-known first order Born and Rytov ones described in Chapter 2, valid for weakly scattering samples [360]. The applicability and accuracy of the Born and Rytov approximations have been well studied in the literature [359, 110, 120, 361, 362], to cite few. The Born approximation is frequently employed in problems in which the radiation is collected in the backscattering direction, such as ultrasounds and optical coherence tomography, and it is generally considered valid when the phase change is smaller than 2π . On the other hand, the Rytov approximation is typically used when the field is detected in the forward scattering direction, as in ODT, TPM and CT problems, and the approximation is accurate when the

square of the phase gradient is much less than the index contrast divided by the wavelength squared over a period of 2π radians. The approximations are asymptotically equivalent for low-contrast, small objects, while the first order Rytov one allows to enlarge the range of applicability of the Born's to a wider class of samples.

However, both of these descriptions are often not accurate enough for the modelling of the forward problem for high-scattering or thick samples and their use might lead to severely corrupted reconstructions. This is the case of many biological samples, and the problem is particularly present in the field of [ODT](#). Many alternative, nonlinear approaches have been developed overtime to provide a more accurate modelling. To name few, beam-propagation methods [[123](#), [117](#), [363](#), [364](#), [365](#), [366](#)] rely on a slice-by-slice propagation model that accounts for multiple scattering happening both in the direction of propagation and backwards. Other nonlinear models include the contrast source-inversion method [[367](#)] or the recursive Born approximation [[368](#)]. In [[369](#)], a sophisticated description based on the Lippmann-Schwinger equation (2.17) was proposed for the modelling of multiple scattering in transmission and reflection for [ODT](#) and proved to be superior to both Born, Rytov and beam-propagation methods. Other recent developments such as the pioneering [[370](#)] integrate [DL](#) approaches, training a physics-informed [DCNN](#) to reproduce the forward scattering calculated by numerical simulators solving for full wave solutions, providing excellent results for the 2D case.

Another class of methods regard the use of higher order scattering modellings. As mentioned in Chapter 2, the first order Born approximation is the first estimate of a high order description, where the field is written as a series of functions proportional to increasingly higher powers of the scattering potential. Accounting for orders successive than the first one improves the quality of the forward modelling and its inversion, as demonstrated in [[371](#), [372](#), [373](#)]. In particular, this was investigated for the second order term of the series [[374](#), [375](#), [376](#), [377](#)]. Considering the Rytov approximation, similar considerations can be made. Indeed, an analogous mathematical description can be made, introducing the Rytov series. Although well-known and formally mathematically described, little literature is present on the use of the Rytov series both for the forward and the inversion problems for real use-case applications [[373](#), [378](#), [120](#)]. Con-

sidering the improvement brought by the first order Rytov approximation with respect to the Born one, it is expected that this would happen also for higher order modellings. Accordingly, the objective of this Chapter will be the mathematical description of the second order Rytov approximation. First, the general solution will be derived using two different approaches, then it will be applied for the first time to the case of scattering from a 2D homogeneous cylinder as a proof of concept, comparing it to the first order Born and Rytov solutions.

5.1 Second order Rytov approximation

The mathematical description here derived is based on the calculations made in [120]; accordingly, the convention used for the expressions is the same as the author and slightly different from the one used in Chapter 2.

The objective of the Rytov approximation is to seek a solution for Eq. (5.1),

$$\begin{aligned} (\nabla^2 + k_m^2)E(\mathbf{r}) &= -f(\mathbf{r})E(\mathbf{r}) \\ f(\mathbf{r}) &= k_m^2 \left[\left(\frac{n(\mathbf{r})}{n_m} \right)^2 - 1 \right] \end{aligned} \quad (5.1)$$

in which $E(\mathbf{r})$ is the total field, $f(\mathbf{r})$ is the scattering potential, k_m the propagation constant in the homogeneous surrounding media, $n(\mathbf{r})$ is the RI profile of the scatterer and n_m is the constant RI of the surrounding media and $\mathbf{r}$ represents the position vector defining a point in random medium. Writing the total field and the incident field $E_0(\mathbf{r})$ in an exponential form as in Eq. (5.2)

$$\begin{aligned} E(\mathbf{r}) &= e^{\psi_0(\mathbf{r}) + \psi(\mathbf{r})} \\ E_0(\mathbf{r}) &= e^{\psi_0(\mathbf{r})} \end{aligned} \quad (5.2)$$

Eq. (5.1) can be mathematically manipulated and rewritten as in Eq. (5.3) following the same passages illustrated in Chapter 2 and exploiting the nomenclature of [120], where $\delta\epsilon$ describes the variation of the dielectric

constant in the media.

$$\begin{aligned}\nabla^2\psi(\mathbf{r}) + (\nabla\psi(\mathbf{r}))^2 + 2\nabla\psi_0(\mathbf{r}) \cdot \nabla\psi(\mathbf{r}) + k^2\delta\epsilon &= 0 \\ E(\mathbf{r}) &= e^{\psi_0(\mathbf{r})+\psi(\mathbf{r})} \\ E_0(\mathbf{r}) &= e^{\psi_0(\mathbf{r})}\end{aligned}\tag{5.3}$$

Eq. (5.3) is a well-known mathematical physics equation called Riccati's equation, whose general form can be expressed as in Eq. (5.4) if we let $\mathbf{U} = \nabla\psi$.

$$\nabla \cdot \mathbf{U} + \mathbf{U} \cdot \mathbf{U} + 2\mathbf{U} \cdot \nabla\psi_0 + k^2\delta\epsilon = 0\tag{5.4}$$

The way to approach Eq. (5.3) according to the Rytov method is to seek for a solution expressed as a series of functions. The terms of this series are proportional to increasingly higher powers of $\delta\epsilon$, as made in the Born series for the electrical field. Accordingly, the phase of the total field ψ is expanded as

$$\psi(\mathbf{r}) = \psi_1(\mathbf{r}) + \psi_2(\mathbf{r}) + \psi_3(\mathbf{r}) + \dots + \psi_n(\mathbf{r}) + \dots\tag{5.5}$$

Forcing Eq. (5.5) into Eq. (5.3), a set of equations for the increasingly higher terms of ψ can be obtained as in Eq. (5.6), where the dependency from $\mathbf{r}$ is omitted for brevity

$$\begin{aligned}\nabla^2\psi_0 + (\nabla\psi_0)^2 + k^2 &= 0 \\ \nabla^2\psi_1 + 2\nabla\psi_0 \cdot \nabla\psi_1 + k^2\delta\epsilon &= 0 \\ \nabla^2\psi_2 + 2\nabla\psi_0 \cdot \nabla\psi_2 + (\nabla\psi_1)^2 &= 0 \\ \nabla^2\psi_3 + 2\nabla\psi_0 \cdot \nabla\psi_3 + 2\nabla\psi_1 \cdot \nabla\psi_2 &= 0\end{aligned}\tag{5.6}$$

In this system, the first equation solved by $\psi_0(\mathbf{r})$ corresponds to an unperturbed situation. The already discussed first order Rytov approximation is obtained manipulating the second equation written in Eqs. (5.6), leading to the well known first order solution here reported

$$\psi_1(\mathbf{R}) = -k^2 \int dr^3 G(\mathbf{R}, \mathbf{r}) \delta\epsilon(\mathbf{r}) \frac{E_0(\mathbf{r})}{E_0(\mathbf{R})}\tag{5.7}$$

in which $\mathbf{R}$ is the vector position of the receiver. Higher order solutions

correspond to successive equations in the system (5.6). The aim in this thesis is to study the second order approximation; hence, a solution for Eq. (5.8) is searched.

$$\nabla^2 \psi_2(\mathbf{r}) + 2\nabla \psi_0(\mathbf{r}) \cdot \nabla \psi_2(\mathbf{r}) + (\nabla \psi_1(\mathbf{r}))^2 = 0 \quad (5.8)$$

In the literature, two approaches have been proposed and discussed in [120]; one developed by V. Tatarskii and another proposed by H. T. Yura and reformulated by Wheelon in [120]. In this thesis, both the expression are discussed, pointing out that their equivalence has been rigorously proven in [120] and numerically by our implementation.

5.1.1 Tatarskii's solution

Tatarskii approach for the solution of Eq. (5.8) starts from the consideration that the equation shares the same mathematical form as Eq. (2.35) and that the right term does not represent an unknown of the problem, as it is the solution of the first order approximation in Eq. (5.7).

$$\nabla^2 \psi_2(\mathbf{r}) + 2\nabla \psi_0(\mathbf{r}) \cdot \nabla \psi_2(\mathbf{r}) + (\nabla \psi_1(\mathbf{r}))^2 = 0 \quad (5.9)$$

Accordingly, a solution can be found for parallelism exploiting the Green function as made when solving the first order expression, leading to Eq. (5.10).

$$\psi_2(\mathbf{R}) = - \int d\mathbf{r}^3 G(\mathbf{R}, \mathbf{r}) (\nabla \psi_1(\mathbf{r}))^2 \frac{E_0(\mathbf{r})}{E_0(\mathbf{R})} \quad (5.10)$$

Which allows us to write the expression for the electric field with a second order Rytov approximation as

$$\begin{aligned} E(\mathbf{R}) &= E_0(\mathbf{R}) e^{[\psi_1(\mathbf{R}) + \psi_2(\mathbf{R})]} = \\ &= E_0(\mathbf{R}) e^{- \int d\mathbf{r}^3 G(\mathbf{R}, \mathbf{r}) [k^2 \delta\epsilon(\mathbf{R}) + (\nabla \psi_1(\mathbf{r}))^2] \frac{E_0(\mathbf{r})}{E_0(\mathbf{R})}} \end{aligned} \quad (5.11)$$

This solution, even though intuitive, is less robust to noise rejection in a numerical implementation given the presence of the squared gradient of the first order solution in the exponential of the electric field.

5.1.2 Yura's solution

The starting point to this alternative solution is again the second order as in Eq. (5.9), where again the ψ_1 term represents the first order solution. Yura proposes to seek for a solution ψ_2 written as

$$\psi_2 = B_2 - \frac{1}{2}B_1^2 \quad (5.12)$$

Forcing this expression into Eq. (5.9) it is possible to write

$$\nabla^2(B_2 - \frac{1}{2}B_1^2) + 2\nabla\psi_0 \cdot \nabla(B_2 - \frac{1}{2}B_1^2) = -(\nabla B_1)^2 \quad (5.13)$$

Developing mathematically this expression and exploiting the second equation in the system (5.6), having identified $B_1 = \psi_1$, it results

$$\nabla^2 B_2 + 2\nabla\psi_0 \nabla B_2 = -k^2 \delta\epsilon \psi_1 \quad (5.14)$$

and again, exploiting the mathematical equivalence to Eq. (5.7), we can write

$$B_2(\mathbf{R}) = -k^2 \int d^3\mathbf{r}_1 G(\mathbf{R}, \mathbf{r}_1) \frac{E_0(\mathbf{r}_1)}{E_0(\mathbf{R})} \delta\epsilon(\mathbf{r}_1) \psi_1(\mathbf{r}_1) \quad (5.15)$$

Substituting the expression of $\psi_1(\mathbf{r}_1)$ as in Eq. (5.7) we get

$$B_2(\mathbf{R}) = k^4 \int d^3\mathbf{r}_1 G(\mathbf{R}, \mathbf{r}_1) \delta\epsilon(\mathbf{r}_1) \int d^3\mathbf{r}_2 G(\mathbf{r}_1, \mathbf{r}_2) \delta\epsilon(\mathbf{r}_2) \frac{E_0(\mathbf{r}_2)}{E_0(\mathbf{R})} \quad (5.16)$$

This expression represents the second term of the Born series, describing double scattering of the incident wave normalized by the unperturbed term as illustrated in Eq. (2.3). Finally, the electric field strength written for the second order Rytov approximation according to the Yura's formulation is expressed as

$$E_2(\mathbf{R}) = E_0(\mathbf{R}) e^{[B_1(\mathbf{R}) + B_2(\mathbf{R}) - \frac{1}{2}B_1(\mathbf{R})^2]} \quad (5.17)$$

The advantage in the use of the described Yura's version is that there is no explicit use of the gradient of the first order solution $\psi_1(\mathbf{r})$ as it happens

in the Tatarskii's version. This allows the Yura's formulation to be more robust with respect to noise in a numerical implementation.

5.2 Second order Rytov for a 2D homogeneous cylinder

To assess the beneficial effects of incorporating a higher order modelling for the scattering problem, the presented description was applied to the problem of the scattering of a linearly polarized plane wave impinging over a 2D homogeneous cylinder. Indeed, in this case it is possible to derive analytically the expressions for the Born, the Rytov approximations at first and second order and to compare them to the full wave solution written through the cylindrical harmonics expansion. This derivation represents the main contribution of this Chapter.

5.2.1 Analytical derivation of the Born, Rytov first order and Rytov second order approximations

The cylinder has radius a and the incident plane wave $U_i(\rho)$, propagating in the x direction and directed along the y direction, is impinging at incidence angle ϕ_i . The geometry of the problem is sketched in Eq. (5.1). The Green function in this case is defined as

$$G(\boldsymbol{\rho}, \boldsymbol{\rho}') = \frac{j}{4} H_0^{(1)}(k_0 |\boldsymbol{\rho} - \boldsymbol{\rho}'|) \quad (5.18)$$

in which the variables marked as prime represent inner points of the cylinder. The solution in every point of the space in a cylindrical coordinate system (ρ, ϕ) must obey the Lippmann-Schwinger equation introduced in

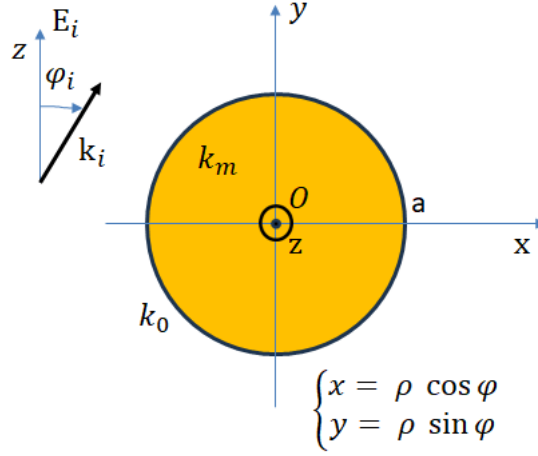


Figure 5.1. Sketch of the scattering problem considered.

Eq. (2.17) and here reported

$$\begin{aligned}
 U(\boldsymbol{\rho}) &= U_i(\boldsymbol{\rho}) + \int_V dx' dy' G(\boldsymbol{\rho}, \boldsymbol{\rho}') f(\boldsymbol{\rho}') U(\boldsymbol{\rho}') \\
 f(\boldsymbol{\rho}) &= k_0^2 (n^2(\boldsymbol{\rho}) - 1) = f_0 \\
 U_i(\boldsymbol{\rho}) &= e^{jk_0 \rho \cos(\alpha)} \\
 \alpha &= \phi - \phi_i
 \end{aligned} \tag{5.19}$$

To manipulate this expression, the starting point is to incorporate the Graph addition theorem which allows to decouple the dependence from the difference between $\boldsymbol{\rho}$ and $\boldsymbol{\rho}'$ in the Green function

$$H_0^{(1)}(k_0 |\boldsymbol{\rho} - \boldsymbol{\rho}'|) = \begin{cases} \sum_{n=-\infty}^{+\infty} H_n^{(1)}(k_0 \boldsymbol{\rho}) J_n(k_0 \boldsymbol{\rho}') e^{jn(\phi - \phi_i)} & \boldsymbol{\rho} > \boldsymbol{\rho}' \\ \sum_{n=-\infty}^{+\infty} J_n(k_0 \boldsymbol{\rho}) H_n^{(1)}(k_0 \boldsymbol{\rho}') e^{jn(\phi - \phi_i)} & \boldsymbol{\rho}' > \boldsymbol{\rho} \end{cases} \tag{5.20}$$

Forcing Eq. (5.20) into Eq. (5.19), outside the cylinder for $\rho > a$ the equation is rewritten as

$$U(\boldsymbol{\rho}) = e^{jk_0\rho\cos(\alpha)} + \frac{j}{4}f_0 \sum_{n=-\infty}^{\infty} H_n^{(1)}(k_0\rho) \int_0^{2\pi} \int_0^a d\boldsymbol{\rho}' \rho' d\phi' J_n(k_0\rho') e^{jn(\phi-\phi')} U(\boldsymbol{\rho}') \quad (5.21)$$

For the case $\rho < a$ both cases of the Graph's theorem must be considered, given the relative position of the source points (ρ', ϕ') and the points where the field is calculated (ρ, ϕ) . Accordingly, the resulting expression is

$$U(\boldsymbol{\rho}) = e^{jk_0\rho\cos(\alpha)} + \frac{j}{4}f_0 \sum_{n=-\infty}^{\infty} H_n^{(1)}(k_0\rho) \int_0^{2\pi} \int_0^\rho d\boldsymbol{\rho}' \rho' d\phi' J_n(k_0\rho') e^{jn(\phi-\phi')} U(\boldsymbol{\rho}') + \frac{j}{4}f_0 \sum_{n=-\infty}^{\infty} J_n(k_0\rho) \int_0^{2\pi} \int_\rho^a d\boldsymbol{\rho}' \rho' d\phi' H_n^{(1)}(k_0\rho') e^{jn(\phi-\phi')} U(\boldsymbol{\rho}') \quad (5.22)$$

To derive the first order Born approximation, the total field in the Lippmann-Schwinger equation is substituted by the incident field. Starting from Eq. (5.21) for the external field it is possible to write

$$U(\boldsymbol{\rho}) = e^{jk_0\rho\cos(\alpha)} + \frac{j}{4}f_0 \sum_{n=-\infty}^{\infty} H_n^{(1)}(k_0\rho) \int_0^{2\pi} \int_0^a d\boldsymbol{\rho}' \rho' d\phi' J_n(k_0\rho') e^{jn(\phi-\phi')} e^{jk_0\rho'\cos(\alpha')} \quad (5.23)$$

The integral over the angular variable ϕ'

$$\int_0^{2\pi} d\phi' e^{jn(\phi-\phi')} e^{jk_0\rho'\cos(\alpha')} \quad (5.24)$$

can be approached considering the variable change $\phi'' = \phi - \phi_i$ and incorporating the well-known solution for the integrals of the cylinder harmonics

reported in [379]

$$\begin{aligned}
 \int_{-\phi_i}^{2\pi-\phi_i} d\phi'' e^{jn(\phi-\phi_i)} e^{-jn\phi''} e^{jk_0\rho'\cos(\phi'')} &= \\
 &= 2\pi j^{|n|} J_{|n|}(k_0\rho') e^{jn(\phi-\phi_i)} = \\
 &= 2\pi e^{jn(\phi-\phi_i)} (-1)^{k(n)} j^{|n|} J_{|n|}(k_0\rho') \quad (5.25) \\
 k(n) &= \begin{cases} 0 & n \geq 0 \\ n & n < 0 \end{cases}
 \end{aligned}$$

Incorporating this result into Eq. (5.21) we obtain

$$\begin{aligned}
 U_{1,Born}(\boldsymbol{\rho}) &= e^{jk_0\rho\cos(\alpha)} + \\
 \frac{j}{4} f_0 2\pi \sum_{n=-\infty}^{\infty} H_n^{(1)}(k_0\rho) (-1)^{k(n)} j^{|n|} e^{jn(\phi-\phi_i)} \int_0^a d\rho' \rho' d\phi' J_n^2(k_0\rho') \quad (5.26)
 \end{aligned}$$

And finally, again considering [379]

$$\int_0^a d\rho' \rho' d\phi' J_n^2(k_0\rho') = \frac{a^2}{2} [J_n^2(k_0a) - J_{n-1}(k_0a)J_{n+1}(k_0a)] \quad (5.27)$$

The analytical expression for the total field written under the Born approximation for the studied case is derived as in Eq. (5.28).

$$\begin{aligned}
 U_{1,Born}(\boldsymbol{\rho}) &= e^{jk_0\rho\cos(\alpha)} + U_{S,1Born}(\boldsymbol{\rho}) = \\
 e^{jk_0\rho\cos(\phi-\phi_i)} + \frac{j\pi}{4} f_0 a^2 \sum_{n=-\infty}^{\infty} (-1)^{k(n)} j^{|n|} H_n^{(1)}(k_0\rho) e^{jn(\phi-\phi_i)} * \quad (5.28) \\
 [J_n^2(k_0a) - J_{n-1}(k_0a)J_{n+1}(k_0a)]
 \end{aligned}$$

Eq. (5.22) for the case of the internal field can be mathematically approached similarly. The first integral term is solved as for the external

case just described

$$\begin{aligned} \frac{j}{4} f_0 \sum_{n=-\infty}^{\infty} H_n^{(1)}(k_0 \rho) \int_0^{2\pi} \int_0^{\rho} d\rho' \rho' d\phi' J_n(k_0 \rho') e^{jn(\phi-\phi')} e^{jk_0 \rho' \cos(\alpha')} = \\ \frac{j\pi}{2} f_0 \left[\frac{\rho^2}{2} \sum_{n=-\infty}^{\infty} (-1)^{k(n)} j^{|n|} H_n^{(1)}(k_0 \rho) e^{jn(\phi-\phi_i)} * \right. \\ \left. [J_n^2(k_0 \rho) - J_{n-1}(k_0 \rho) J_{n+1}(k_0 \rho)] \right] \end{aligned} \quad (5.29)$$

While the second integral term

$$\begin{aligned} \frac{j}{4} f_0 \sum_{n=-\infty}^{\infty} J_n(k_0 \rho) * \\ \int_0^{2\pi} \int_{\rho}^a d\rho' \rho' d\phi' H_n^{(1)}(k_0 \rho') e^{jn(\phi-\phi')} e^{jk_0 \rho' \cos(\alpha')} \end{aligned} \quad (5.30)$$

can be approached making the same consideration for the angular integration as in Eq. (5.25) and noting that the integral

$$\begin{aligned} \int dx x J_n^{(1)}(k_0 x) H_n^{(1)}(k_0 x) = g(n, k_0, x) \\ g(n, k_0, x) = \frac{x^2}{4} [2J_n(k_0 x) H_n^{(1)}(k_0 x) + \\ - J_{n-1}(k_0 x) H_{n+1}^{(1)}(k_0 x) - J_{n+1}(k_0 x) H_{n-1}^{(1)}(k_0 x)] \end{aligned} \quad (5.31)$$

has a well known solution [379]. It is worth mentioning for a numerical implementation that the function $g(n, k_0, x)$ is singular for the value $x = 0$. In this point, the value is assigned as

$$\lim_{x \rightarrow 0} g(n, k_0, x) = \frac{j|n|}{\pi k_0^2} \quad (5.32)$$

Merging Eqs. (5.31) and (5.25) into Eq. (5.30), an expression for the second integral term can be obtained

$$\frac{jf_0\pi}{2} \sum_{n=-\infty}^{\infty} J_n(k_0\boldsymbol{\rho})(-1)^{k(n)}j^{|n|}e^{jn(\phi-\phi_i)}_* \quad (5.33)$$

$$[g(n, k_0, a) - g(n, k_0, \boldsymbol{\rho})]$$

and therefore the full expression for the Born approximation for the internal field is written as

$$U_{1,Born}(\boldsymbol{\rho}) = e^{jk_0\rho\cos(\phi-\phi_i)} +$$

$$\frac{j\pi}{4}f_0a^2 \sum_{n=-\infty}^{\infty} (-1)^{k(n)}j^{|n|}H_n^{(1)}(k_0\boldsymbol{\rho})e^{jn(\phi-\phi_i)}_*$$

$$[J_n^2(k_0\boldsymbol{\rho}) - J_{n-1}(k_0\boldsymbol{\rho})J_{n+1}(k_0\boldsymbol{\rho})] + \quad (5.34)$$

$$+ \frac{jf_0\pi}{2} \sum_{n=-\infty}^{\infty} J_n(k_0\boldsymbol{\rho})(-1)^{k(n)}j^{|n|}e^{jn(\phi-\phi_i)}_*$$

$$[g(n, k_0, a) - g(n, k_0, \boldsymbol{\rho})]$$

Once the Born approximation has been derived, the expression for the Rytov approximation comes straightforwardly considering the relation between the two described in Eq. (2.36). Accordingly, for the external field it is possible to write

$$U_{1,Rytov}(\boldsymbol{\rho}) = U_i(\boldsymbol{\rho})e^{\{(\frac{U_{S,1Born}}{U_i(\boldsymbol{\rho})})\}} \quad (5.35)$$

Whereas the Rytov phase for the internal field can be written as

$$\psi_{1,Rytov}(\boldsymbol{\rho}) = \frac{\pi jf_0}{2U_i(\boldsymbol{\rho})}[\frac{\rho^2}{2} \sum_{n=-\infty}^{\infty} (-1)^{k(n)}j^{|n|}H_n^{(1)}(k_0\boldsymbol{\rho})e^{jn(\phi-\phi_i)}_*$$

$$[J_n^2(k_0\boldsymbol{\rho}) - J_{n-1}(k_0\boldsymbol{\rho})J_{n+1}(k_0\boldsymbol{\rho})] + \quad (5.36)$$

$$+ \frac{jf_0\pi}{2} \sum_{n=-\infty}^{\infty} J_n(k_0\boldsymbol{\rho})(-1)^{k(n)}j^{|n|}e^{jn(\phi-\phi_i)}_*$$

$$[g(n, k_0, a) - g(n, k_0, \boldsymbol{\rho})]]$$

For the second order solution, the approach developed by Yura is used. As discussed above, the solution is composed of two pieces, B_1 and B_2 , whose expressions have been derived in the previous section. Forcing the derived expression of $\psi_{1,Rytov}$ into Eq. (5.15) we get

$$\begin{aligned}
 B_2(\boldsymbol{\rho}) = \frac{jf_0}{4U_i(\boldsymbol{\rho})} \frac{\pi jf_0}{2} \sum_{n,m=-\infty}^{\infty} \int_V d\phi' d\boldsymbol{\rho}' \boldsymbol{\rho}' H_n(1)(k_0\boldsymbol{\rho}) J_n(k_0\boldsymbol{\rho}') e^{jn(\phi-\phi')} * \\
 (-1)^{k(m)} j^{|m|} e^{jm(\phi'-\phi_i)} [H_m(1)(k_0\boldsymbol{\rho}') \frac{\boldsymbol{\rho}'^2}{2} * \\
 [J_m^2(k_0\boldsymbol{\rho}') - J_{m-1}(k_0\boldsymbol{\rho}') J_{m+1}(k_0\boldsymbol{\rho}')] + \\
 + J_m(k_0\boldsymbol{\rho}') [g(m, k_0, a) - g(m, k_0, \rho')]]
 \end{aligned} \quad (5.37)$$

for brevity, defining

$$\begin{aligned}
 A = [H_m^{(1)}(k_0\boldsymbol{\rho}') \frac{\boldsymbol{\rho}'^2}{2} [J_m^2(k_0\boldsymbol{\rho}') - J_{m-1}(k_0\boldsymbol{\rho}') J_{m+1}(k_0\boldsymbol{\rho}')] + \\
 + J_m(k_0\boldsymbol{\rho}') [g(m, k_0, a) - g(m, k_0, \rho')]]
 \end{aligned} \quad (5.38)$$

and following the calculations,

$$\begin{aligned}
 B_2(\boldsymbol{\rho}) = \frac{-f_0^2 \pi}{8U_i(\boldsymbol{\rho})} \sum_{n,m=-\infty}^{\infty} H_n^{(1)}(k_0\boldsymbol{\rho}) (-1)^{k(m)} j^{|m|} * \\
 \int_0^{2\pi} d\phi' e^{jn(\phi-\phi')} e^{jm(\phi-\phi_i)} \int_0^a d\boldsymbol{\rho}' \boldsymbol{\rho}' J_n(k_0\boldsymbol{\rho}') A
 \end{aligned} \quad (5.39)$$

if we let

$$\begin{aligned}
 A_2 = \int_0^a d\boldsymbol{\rho}' \boldsymbol{\rho}' J_n(k_0\boldsymbol{\rho}') A = \\
 \int_0^a d\boldsymbol{\rho}' \boldsymbol{\rho}' [J_n(k_0\boldsymbol{\rho}') H_n^{(1)}(k_0\boldsymbol{\rho}') \frac{\boldsymbol{\rho}'^2}{2} [J_m^2(k_0\boldsymbol{\rho}') - J_{m-1}(k_0\boldsymbol{\rho}') J_{m+1}(k_0\boldsymbol{\rho}')] + \\
 + J_m(k_0\boldsymbol{\rho}') J_n(k_0\boldsymbol{\rho}') g(m, k_0, a) - J_m(k_0\boldsymbol{\rho}') J_n(k_0\boldsymbol{\rho}') g(m, k_0, \rho')]
 \end{aligned} \quad (5.40)$$

then

$$B_2(\boldsymbol{\rho}) = \frac{-f_0^2 \pi}{8U_i(\boldsymbol{\rho})} \sum_{n,m=-\infty}^{\infty} H_n^{(1)}(k_0 \boldsymbol{\rho}) (-1)^{k(m)} j^{|m|} e^{jn\phi_*} e^{-jm\phi_i} \int_0^{2\pi} d\phi' e^{j(m-n)\phi'} A_2 \quad (5.41)$$

And considering

$$\int_0^{2\pi} d\phi' e^{j(m-n)\phi'} = \delta_{(m,n)} 2\pi \quad (5.42)$$

Eq. (5.43) can finally be obtained,

$$B_2(\boldsymbol{\rho}) = \frac{-f_0^2 \pi^2}{4U_i(\boldsymbol{\rho})} \sum_{n=-\infty}^{\infty} H_n^{(1)}(k_0 \boldsymbol{\rho}) (-1)^{k(n)} j^{|n|} e^{jn(\phi-\phi_i)} * A_3 \quad (5.43)$$

Where

$$\begin{aligned} A_3 = \int_0^a d\boldsymbol{\rho}' \boldsymbol{\rho}' J_n(k_0 \boldsymbol{\rho}') H_n^{(1)}(k_0 \boldsymbol{\rho}') \frac{\boldsymbol{\rho}'^2}{2} [J_n^2(k_0 \boldsymbol{\rho}') - J_{n-1}(k_0 \boldsymbol{\rho}') J_{n+1}(k_0 \boldsymbol{\rho}')] + \\ + \int_0^a d\boldsymbol{\rho}' \boldsymbol{\rho}' J_n(k_0 \boldsymbol{\rho}')^2 g(n, k_0, a) + \\ - \int_0^a d\boldsymbol{\rho}' \boldsymbol{\rho}' J_n(k_0 \boldsymbol{\rho}')^2 g(n, k_0, \boldsymbol{\rho}') \end{aligned} \quad (5.44)$$

and

$$B_1(\boldsymbol{\rho}) = -\frac{1}{2} \psi_{1,Rytov}^2(\boldsymbol{\rho}) \quad (5.45)$$

5.2.2 Numerical Validation

To quantitatively compare the different approximation, the derived analytical expressions were implemented using Wolfram Mathematica. The ground truth scattered field, referred to as full wave solution, was computed making use of the cylindrical harmonics expansion as described in

[380]. The plane wave impinges at an angle $\phi_i = 0$. Several cases were considered. In the first one, the considered cylinder had radius 1λ , while in the second one the cylinder had radius 5λ . Both the cases were tested for two values of RI values, specifically $n = 1.005$, leading to $\epsilon = n^2 = 1.01$, and $n = 1.05$, leading to $\epsilon = n^2 = 1.1$. The scattered field is computed for a fixed x distance equal to x_D . When the radius of the cylinder was set to 1λ , then $x_D = 8\lambda$, while when the radius was set to 5λ , then $x_D = 12\lambda$. The y values at which the scattered field was computed were equal to $[-x_D, x_D]$ with a step of $\frac{\lambda}{10}$. Figs. 5.2 and 5.3 report the results for the two cases of radius of the cylinder, showing the absolute values and the phase, wrapped and unwrapped, of the reconstructed fields. Table 5.1 reports the normalised rms% computed between the Born and the two Rytov approximations respect the full wave solution. As can be seen by the Figs. and the Table, the second order Rytov allows an improvement of at least one order of magnitude with respect to the first order Rytov and the Born approximation almost in every case. This is particularly evident for the cases of greater RI and radius values, where the higher order Rytov solution allows to follow more closely the full wave shape. The final presented case (radius = 5λ and $n = 1.05$) is a challenging one and none of the approximations allow a good representation of the field, in particular for the phases. In particular, the Born approximation completely fails in the representation, while comparing the first and the second order Rytov, a decise improvement is still brought by the high order solution.

rms%, normalised	Born	Rytov 1	Rytov 2
RI = 1.005, $r = 1\lambda$	2.91 %	2.18 %	0.05 %
RI = 1.05, $r = 1\lambda$	29.38 %	21.79 %	4.95 %
RI = 1.005, $r = 5\lambda$	14.11 %	2.51 %	0.18 %
RI = 1.05, $r = 5\lambda$	158.62 %	33.91 %	25.46 %

Table 5.1. rms% normalized computed between the scattered field obtained with different approximations (Born, Rytov 1st. and 2nd. order) and the full wave solution.

Lastly, an more complete analysis was conducted to numerically investigate the range of validity of the 2nd order Rytov approximation. In particular, first the radius of the cylinder was fixes at $r = 1\lambda$ and the detector distance x_D at 10λ , while the RI of the scatterer was changed from 1.005 to 1.05

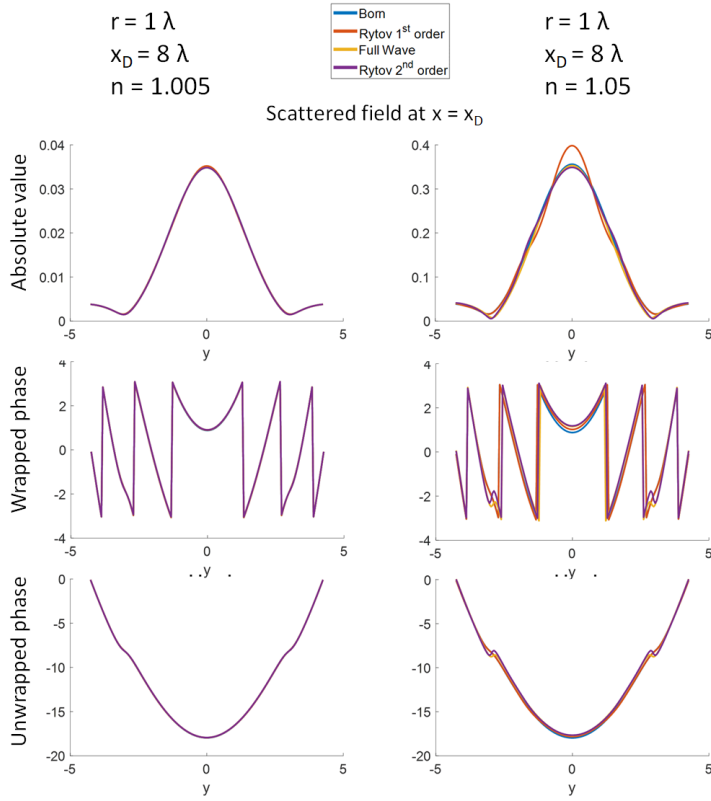


Figure 5.2. Scattered field reconstructed at the detector. Comparison between the Born, Rytov 1st and 2nd order and the full wave solutions for a cylinder of radius 1λ .

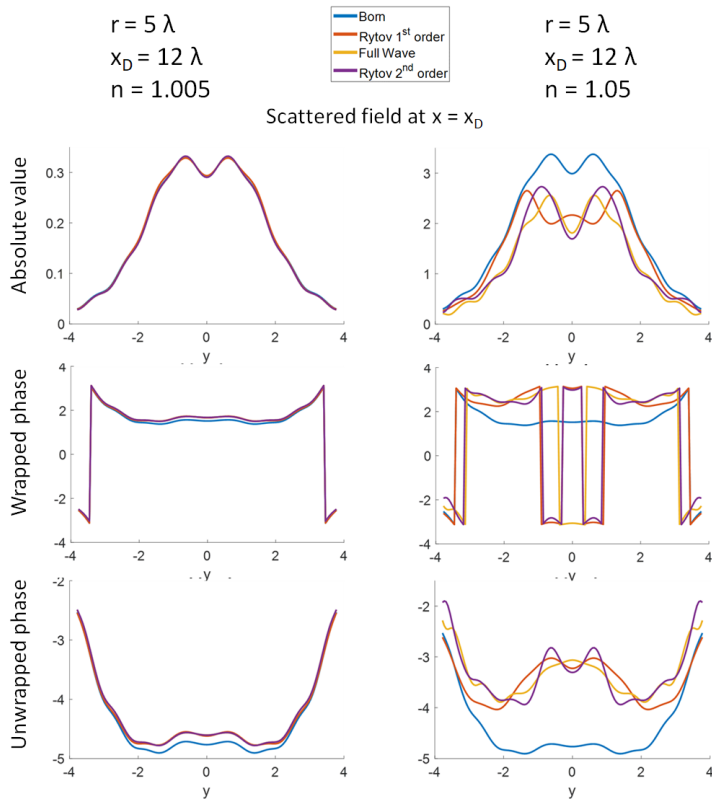


Figure 5.3. Scattered field reconstructed at the detector. Comparison between the Born, Rytov 1st and 2nd order and the full wave solutions for a cylinder of radius 5λ .

with a step of 0.05. The results are reported in Fig. 5.4, where the rms% normalised evaluated between the various approximation and the full wave solutions are reported for each RI value. The beneficial effect of the higher order modelling can be appreciated, with an rms% always below the 5%.

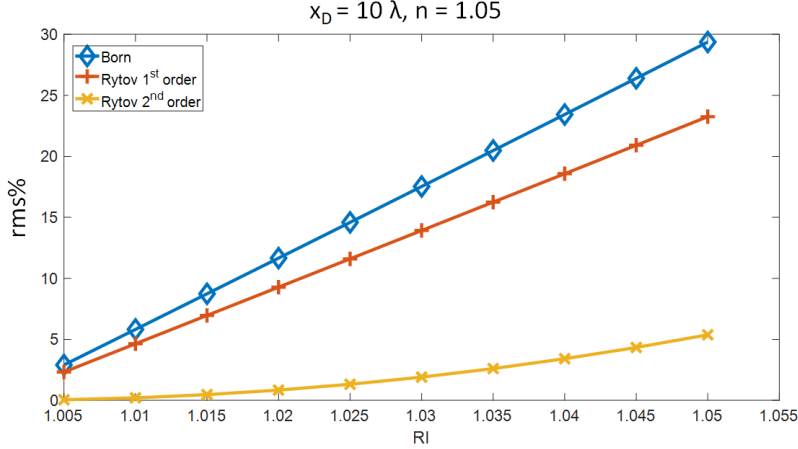


Figure 5.4. Rms% normalized computed between the various approximations and the full wave solution as a function of the RI of the cylinder.

In Fig. 5.5, the results of a second analysis are shown. In this case, the RI value was set equal to 1.05 and the recording distance x_D was kept equal to 10λ , while the radius of the cylinder increased from 1λ to 5λ with a step of 0.5λ . This case represents a more challenging study, as confirmed by the high values of rms% normalised computed between the various approximations and the full wave solution and reported in subfigure (a), and it is useful to better define the range of validity of the approximation. Nonetheless, even though the values of rms% for the two Rytov approximations get closer as the radius of the cylinder increases, looking at subfigure (b) it can be observed that the higher order modelling helps representing the phase of the scattered field, which is generally the sought after information in applications such as TPM, confirming the importance of a higher order modelling.

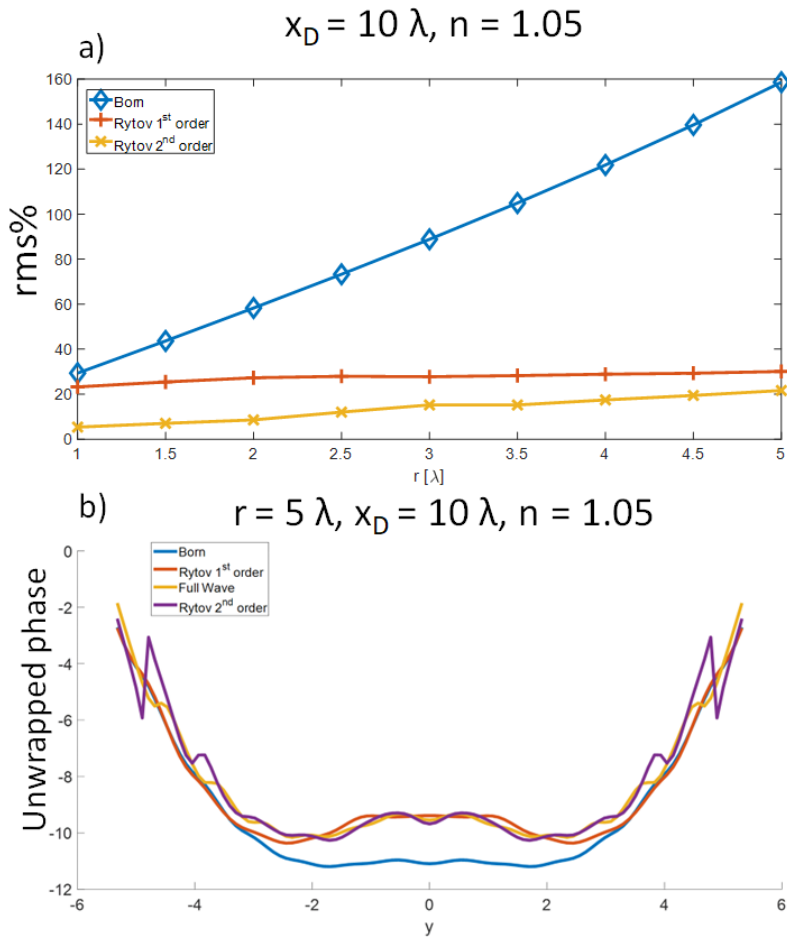


Figure 5.5. Comparison between the various approximations as a function of the radius of the cylinder. (a) rms% normalised computed between the various approximations and the full wave solution as a function of the radius of the cylinder. (b) Unwrapped phases for $RI = 1.05$ and $r = 5 \lambda$.

Conclusions

This Ph.D. Thesis aimed to discuss the paradigm of Computational Tomography and to overcome some of the numerical issue associated to it. Starting from the first proof of concept of [TPIFC](#), many aspects of the conversion of this technology toward [LOC](#) applications have been discussed, proposing solution aiming to tackle some of the major issues associated.

- From the technological and numerical point of view, the feasibility of a common path setup for [TPIFC](#) has been demonstrated, and an adequate numerical tool based on [HOTV](#) apt to the processing of data acquired from the mentioned setup has been successfully integrated for the first time in this context [225]. The proposed numerical approach allows to obtain tomographic reconstructions significantly improved with respect to the standard tomographic solver ([FBP](#)), as well as to the conventional [TV](#) method. The future developments of this technique are currently under investigation, with the possibility to introduce fractional calculus into the reconstruction.
- An extremely fast and lightweight [CNN](#) has been used to enhance the quality of the 2D reconstructions obtained with [FBP](#). The developed [CNN](#) has a particularly low inference time, comparable to the desired speed of [FBP](#) and 60% faster than the state of the art [DL](#)-based solvers, representing a powerful reconstruction method for tomographic data collected with a reduced number of orientations [289]. A full 3D version of this method is now under development

and will constitute the further evolution of the proposed DL approach.

- AI has been successfully integrated for the development of a method to discriminate between healthy and cancer cells. The studied classifiers, thanks to their properties of minimising the number of obtained false negative, constitute a preliminary tool for LB-based applications, mainly focusing on the early-stage detection of tumours [340].
- A CNN has been proposed as an evolution of [340], allowing to robustly classify cells into several classes starting from multiple acquired QPMs relative to different orientations. In this work, it was demonstrated that the availability of angular-dependent information, even though non-fully extracted through tomography, strongly increments the results of the classification without the need to resort to computationally heavy tomographic reconstruction [356].
- A preliminary study regarding higher order modellings of the scattering phenomena has been conducted. In particular, an analytical expression for the second order Rytov scattering has been developed for the case of an homogeneous 2D cylinder and compared to the first order Born and Rytov approximations, underlying the strong benefits it brings in the description. This study paves the way to the embedding of more accurate scattering theory into the tomographic problem, which might lead to the availability of more robust inversion solvers. The further application of this description also to non-homogeneous structures, the development of a related inversion technique and the extension of this work to a 3D framework will constitute the next advancements of this explorative study.

The intrinsic technological nature of TPIFC, combined to the obtained results, make it eligible for introducing a breakthrough in several clinical applications based on label-free single-cell analysis. In fact, thanks to the potential high-throughput modality and the high informative contents of the 3D RI tomogram, the developed computational methodologies allow the TPIFC to offer a practical solution in the next future of telemedicine applications at LOC scale.

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