Review

Imaging Flow Cytometry: Development, Present Applications and Future Challenges

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Imaging Flow Cytometry: Development, Present Applications and Future Challenges

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Abstract: Imaging flow cytometry (ImFC) represents a significant technological advancement in the field of cytometry, effectively merging the high-throughput capabilities of flow analysis with the image-based features of microscopy. In this comprehensive review article, we take a historical perspective and delve into the evolution of this methodology, presenting its past, exploring current state-of-the-art and forecasting potential future advancements. The inception of ImFC arose from the innovative combination of a flow cytometer's hydraulics with the technology of an advanced camera. This synergistic coupling facilitated the morphological analysis of cell populations at a high-throughput scale, effectively evolving the landscape of cytometry. However, the application of ImFC faced several challenges. Among the most prominent ones, is the development and implementation of software systems that can reliably and efficiently handle the robust data acquisition and analysis inherent in ImFC. The scale and complexity of the data generated by ImFC necessitates the creation of novel analytical tools that can effectively manage and interpret this data, thus allowing us to unlock the full potential of ImFC. Interestingly, artificial intelligence (AI) algorithms have begun to be implemented on ImFC, offering a promise for enhancing analytical capabilities. The adaptability and learning capacity of AI may prove to be essential in knowledge mining from high-dimensional data produced by ImFC, potentially enabling more accurate analyses. Looking forward, we project that ImFC may become an indispensable tool, not only in research laboratories but also in clinical settings. Given the unique combination of high-throughput cytometry and detailed imaging offered by ImFC, we foresee a critical role for this technology in the next generation of scientific research and diagnostics. As such, we encourage both current and future scientists to consider the integration of ImFC as an addition to their research toolkit and clinical diagnostic routine.

Keywords: flow cytometry; imaging flow cytometry; artificial intelligence; phenotype analysis

1. Introduction

Flow cytometry (FC) is a powerful methodology for characterization of complex phenotypes in cellular populations as well as the quantification of cellular processes such as proliferation, cell death and cell differentiation [1]. The rationale of FC is based on analysis of spectral characteristics of cell in a homogeneous liquid mixture. A standard flow cytometer, in which FC analysis is performed is into three distinct systems: A hydraulics system, an optical system and an electronics system. The hydraulics system performs hydrodynamic focusing in order for cells to pass sequentially, as single events, through an interrogation point. In this point, as part of the optical system, a laser source is used for the excitation specific fluorophores (that have been added in a preanalytical step), while the emitted fluorescence from each cell is passed through detection filters. Third, the electronics system stores fluorescence signals in a digital format [1–4]. These characteristics make flow cytometry invaluable in several scientific fields such as hematology, immunology and oncology, among others [5–15].

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Imaging flow cytometry (ImFC) stands as an innovative and technologically advanced offshoot of conventional flow cytometry (FC), distinguished by new modifications and improvements to existing techniques Using the camera, ImFC is able to provide granular and high-quality visual information about the detected cells. More specifically, it provides broad information about the morphology, or structural characteristics, of the cell. This includes detailed analysis of cell size, shape, internal structure, and the distribution of specific components or markers within the cell. Thus, the combination of imaging technologies with flow cytometry represents a major advance in cellular analysis [16]. In that way, the user has a supplementary source of information to validate fluorescence data and to provide a comprehensive and accurate characterization on cell populations.

ImFC has the advantages of both flow cytometry and microscopy. First, it is advantageous over typical FC in a manner that provides structural information apart from the spectral properties of the cells. Second, it provides high-throughput, a feature that is not available in fluorescence microscopy [17]. The current review unfolds the chronological progression of Imaging Flow Cytometry (ImFC), from its historical roots to its contemporary applications, and looks forward to potential challenges in its future deployment. It also critically addresses the pivotal steps required to transition this technology from laboratory research to practical clinical use.

2. History of Imaging flow Cytometry

FC development through the years is based on the same principles as a fluorescence microscope, with the main difference being that the fluorescence is digitalized as a signal and not as an image [1]. Following the vision that a cytometer could employ imaging capabilities, an initial implementation of ImFC has been developed as early as 1978, by a research team in Rochester University that envisioned a a cytometer could utilize imaging capabilities, leading to the initial development of Imaging Flow Cytometry [18,19]. The authors developed a slit-scan flow system that performed slit-imaging. This was a preliminary technological milestone that was however ahead of its time that needed a necessary technology evolution that took more than 20 years. Critical technology implementations in detectors and imaging systems led to the first commercially available Imaging Flow Cytometers [20]. Commercial systems, even though lacked resolution compared to fluorescence microscopes allowed the analysis of protein particles [21]. These implementations, though promising, never commercialized and the field remained hindered for nearly two decades, mainly because new technologies were in need to support ImFC requirements.

For the next two decades, the scientific and technological community worked with refining and enhancing this innovative technique. During this period, the major hurdles stemmed from the limitations of then-existing technology in detectors and imaging systems. The process of capturing and interpreting detailed cell images in real-time at the scale needed for flow cytometry required far more sophisticated and powerful hardware and software than those available at that time. Throughout the 1980s and 1990s, significant efforts were made to develop the necessary technological components and increase their resolution and speed while ensuring their reliability. By the turn of the century, research and development in the field began to bear fruit, ultimately leading to the launch of the first commercially available Imaging Flow Cytometers, asd part of several other developments in the field of Cytometry This marked the beginning of ImFC in cellular analysis in parallel to Multiparameter Flow Cytometry [1,22–25].

3. State of the Art: Current Imaging Cytometers and Present applications

The trajectory of Imaging Flow Cytometry (ImFC) saw a marked progression with the first successful introduction of commercial ImFC systems, notably Imagestream and FlowSight, originally developed by Amnis Corporation. These robust systems revolutionized the field with their superior capabilities and advanced features. These intellectual properties were acquired by EMD Millipore in 2011. As of the present day, these systems are marketed and commercialized under the umbrella of Luminex Corporation group. This consolidation represents an important milestone in the commercial trajectory of ImFC systems, highlighting their growing prominence in the scientific and medical industry.

A number of applications of imaging flow cytometry are summarized in Table 1. A very interesting application is the label-free analysis of cell-cycle distribution [26]. High–throughput ImFC

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used to demonstrate a polarized antigen distribution in B cells during an immune response that was sustained among progeny. During the activation of humoral immune responses, B cells acquire antigen for subsequent presentation to cognate T cells. After mouse B cells accumulate antigen, it is maintained in a polarized distribution for extended periods in vivo. Using high-throughput imaging flow cytometry, they observed that this polarization is preserved during B cell division, promoting asymmetric antigen segregation among progeny [27]. In conclusion, high-throughput ImFC has successfully revealed cell cycle distribution of cells as well as a consistent pattern of polarized antigen distribution in B cells during immune responses, a pattern that persists across generations. This technological advancement, thus, plays a pivotal role in enhancing our understanding on the mechanics of immune response activation and its implications on B cell division and subsequent antigen segregation among progeny.

Table 1. Applications of Imaging Flow Cytometry.

Application	**Description**	**Ref.**
Label-free analysis of cell-cycle distribution	ImFC has been used to demonstrate a polarized antigen distribution in B cells during an immune response that was sustained among progeny. This has successfully revealed cell cycle distribution of cells and a consistent pattern of polarized antigen distribution in B cells during immune responses, a pattern that persists across generations.	[26]
Analysis of intracellular pathogens	ImFC has successfully used for analysis of Toxoplasma gondii and Mycobacterium tuberculosis infections in cell lines. This type of analysis offers a prospect of studying host-pathogen dynamic interactions.	[28]
Cell-sorting	ImFC offers the capability of cell-sorting, a feature available in specialized FC sorters. ImFC offered single cell resolution and highly accurate label-free sorting, above 90%, in several experimental conditions.	[30],[31]
microparticle imaging	ImFC has been used for high-throughput single-microparticle imaging flow analysis.	[62]
Ghost cytometry	Ghost cytometry, a technique for classifying cells and other microparticles without the need for labeling or imaging, has been used in conjunction with ImFC. Potential for cell sorting	[64],[65] [66]
Imaging Mass Cytometry	A novel technology that combines flow cytometry and mass spectrometry capabilities using a laser scanning of a tissue sample.	[69]

*Relevant References.

ImFC has been applied in the analysis of intracellular pathogens. Haridas et al. report Imagestream-based analysis of Toxoplasma gondii and Mycobacterium tuberculosis infections in cell lines [28]. This type of analysis offers a prospect of studying host-pathogen dynamic interactions. Significantly, the advantages offered by ImFC support a diagnostic potential with a prospect in

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translation in clinical practice [29]. The capacity to scrutinize host-pathogen interactions, coupled with ImFC's potential diagnostic capabilities, underscores ImFC immense potential for integration into clinical practice, which could revolutionize patient care and infectious disease management.

ImFC offers the capability of cell-sorting, a feature available in specialised FC sorters [30,31]. ImFC offered single cell resolution and highly accurate label-free sorting, above 90%, in several experimental conditions [32]. Label-free cells are easier to manipulate thereafter and do not undergo the preparation step of labeling that may lead to a decrease in viability. The increased accuracy of ImFC to isolate single target cells is believed to be mostly based on the capability of morphological data analysis [32]. The implementation of machine-learning algorithms is considered to further assist in the development highly accurate, label-free cell-sorting [33,34]. The unique capability of ImFC to perform cell-sorting at single-cell resolution, with an impressive accuracy of above 90% in diverse experimental conditions, opens up new frontiers in cell-based research. Notably, its ability to perform label-free sorting, enhanced by morphological data analysis and further augmented with machine-learning algorithms, minimizes the need for preparation steps that could potentially reduce cell viability, thereby simplifying cell manipulation and ensuring a higher degree of cell integrity.

ImFC has made significant strides since its inception, particularly with the introduction of commercial systems like Imagestream and FlowSight. Based on these advancements ImFC has been applied in the label-free analysis of cell-cycle distribution and cellular processes such as polarized antigen distribution in B cells during an immune response. ImFC has also been proven invaluable in studying intracellular pathogens, such as Toxoplasma gondii and Mycobacterium tuberculosis infections and thus indicating a promising avenue for the integration of this technology into clinical practice. Another important feature of ImFC is its cell-sorting capability, providing highly accurate sorting at a single-cell resolution. The implementation of machine-learning algorithms has further refined this process, reducing the need for preparation steps that could compromise cell viability. Collectively, the progress in the field underscores the significant potential of ImFC in advancing cell-based research and clinical, thereby contributing substantially to the medical and scientific industry.

4. Implementation of Machine Learning and Artificial intelligence

Artificial Intelligence (AI) and Machine Learning (ML) represent transformative technologies that are revolutionizing various sectors of society. AI refers to the simulation of human intelligence processes by machines, especially computer systems, while ML, a subset of AI, involves the development of algorithms that allow computers to learn and make decisions from data, thus improving their performance over time without being explicitly programmed. The application of ML algorithms has been widely used in imaging flow cytometry [35]. In a multiplex analysis by Eulenberg et al., deep learning offered the prospect of reconstructing biological processes such as cell cycle distribution as well as disease progression [36]. Importantly, deep-learning based predictions, apart from sensitive, have been proved fast enough to support the high-throughput of generated data from ImFC. A deep learning approach has been succesfully used to assess Imaging flow cytometry data and is believed to change the landscape of high throughput cell analysis [37]. Several successful applications support this notion. For example, 3D imaging flow cytometry (3D-ImFC) was performed by Subramanian R et al., to reveal hepatic stellate cell (HSC) and liver endothelial cell (LEC) morphology at single cell resolution. In their study, was proposed a combination of transmission and side scattered single cell images of liver cells with artificial intelligence to provide a staging system of NASH progression [38]. Furthermore, tomographic imaging flow cytometry (tIFC) arised to prevail over 2D to a 3D imaging of surface and internal structures of particles [39]. The successful integration of AI and ML in imaging flow cytometry exemplifies the exciting prospects of these technologies in advancing our understanding of cell biology and disease mechanisms.

ImFC has proven to be a valuable tool in the field of protein therapeutics, specifically it has been successfully exploited in a study of protein-silicon oil (SO) complexes. These complexes arise when protein particles adsorb onto silicon oil droplets, a process which has the potential to elicit an immune response, thus making it crucial to understand and monitor. In the study by Probst et al. ImFC has provided a unique ability to measure key parameters of these insoluble particles, including their

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number and shape. To further enhance the utility of this technology, researchers have incorporated ML techniques. By using a high amount of particle image data generated by ImFC, they have developed a ML model that can effectively categorize and count protein-SO compounds [40]. This combination of imaging flow cytometry and machine learning not only enhances the analysis of protein-SO complexes but also broadens the scope of future applications in protein therapeutics and other related fields.

Equally prestigious is the contribution of ImFC and artificial intelligence on classification of non-white blood (non-WBC) cells such as circulating rare cells in peripheral blood nucleated cells (PBNCs). Hirotsu et al. combined label-free ImFC with artificial intelligence software and managed to distinguish circulating rare cells as non-WBC fractions among PBNCs in cancer patients and healthy volunteers. The described method offers exciting prospects for cancer patient monitoring and therapy optimization [41].

In a recent article [42], Doan et al. described a workflow of analyzing ImFC images with deep learning methods: after training a model with example images that are known to have a particular phenotype, the model takes the pixels of images as its input and predict an answer for new images. The direct analysis approach was also adopted in a recent study using conventional microscopy, wherein a fluorescent marker of differentiation was used as a ground truth for training a classifier that prospectively predicted cell differentiation from their bright-field images [43].

Deep Cytometry is a recent implementation of deep learning in which image reconstruction is not necessary and real-time, non-supervised cell sorting can be achieved [44]. The future implications of Deep Cytometry are beginning to emerge. First, by employing deep learning algorithms, cell analysis and sorting can be performed in real-time with minimal human intervention. This can dramatically increase the speed and efficiency of the process, enabling the handling of larger samples and potentially leading to more robust results. Second, AI could potentially be used to identify complex patterns or characteristics in cells that may not be immediately apparent to human analysts, based on subtle correlations and features. Third, AI holds promise in predicting future trends or behaviors based on historical data. This could be instrumental in studying disease progression or the effects of treatments at a cellular level. By analyzing past cytometry data using AI, predictive models can be created to forecast cellular responses under certain conditions.

AI, ML and especially deep learning techniques have had a transformative impact on imaging flow cytometry, enabling unprecedented accuracy and efficiency in multicellular data analysis and increasing our understanding of complex biological phenomena such as disease progression and immunity. AI and ML facilitated highly accurate, real-time, unsupervised cell analysis. They also hold a potential to provide valuable insights into complex cell morphology, opening new frontiers in cell-based research, and suggest future prospect for clinical application.

5. Imaging Flow Cytometry and Haematology: Fundamentals for a paradigm shift?

In the previous sections of this review, we have revisited the concept that Imaging Flow Cytometry represents a significant technological advancement in the field of cytometry, by merging the high-throughput capabilities of flow analysis with the image-based features of microscopy. This innovative approach has the potential to open new avenues for the study of haematological diseases, offering distinct advantages over traditional cytometry as well as to complement and/or compete with other novel molecular methodologies such as Next Generation Sequencing (NGS).

NGS has revolutionized haematology, providing insights into the genetic and molecular underpinnings of various blood disorders [45,46]. NGS technologies have enabled comprehensive genomic profiling of haematological malignancies, such as leukemia and lymphoma, revealing complex mutational landscapes that drive these diseases [47–50]. This has not only improved our understanding of disease pathogenesis, but has also facilitated the development of targeted therapies, leading to more personalized and effective treatment strategies and has been instrumental in the detection of measurable residual disease (MRD), a critical factor in patient prognosis and treatment success [51–54]. Despite the challenges associated with data interpretation and integration into

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clinical practice, the impact of NGS in haematology has been transformative, and its potential for further advancements in diagnosis, prognosis, and treatment continues to be immense.

NGS and traditional flow cytometry, while distinct in their methodologies, both play crucial roles in the field of haematology. They complement each other in that flow cytometry provides rapid, real-time analysis of cellular characteristics and protein expression, while NGS offers a deep dive into the genetic and molecular landscape of cells. However, they also compete in certain areas. For instance, while flow cytometry has been the gold standard for immunophenotyping and for measurable residual disease detection, NGS is increasingly being used for such applications since it also offers great sensitivity and specificity in detecting disease-associated genes and low-frequency mutations [55–58].

Beyond classic cytometry, one of the primary advantages of ImFC is its high-throughput capabilities. Unlike fluorescence microscopy, which is limited to process a large number of cells, and NGS, which requires extensive time for sequencing and data analysis, ImFC can rapidly process and analyze several cells per second. This high-throughput capability allows for a more comprehensive analysis of cell populations, which is particularly beneficial in the context of haematological diseases where disease progression can be monitored by changes in cell populations. In addition to its high-throughput capabilities, ImFC provides detailed morphological information about the cells it analyzes, including cell size, shape, internal complexity, and the distribution of specific markers in the cell. This level of detail is not typically available in NGS, which focuses more on genetic and molecular information. In haematological diseases, cellular morphology can provide important diagnostic and prognostic information, making ImFC a valuable tool in these contexts [59,60]. ImFC also leverages the power of deep learning for data analysis. Deep learning-based predictions, apart from being sensitive, have been proven fast enough to support the high-throughput of data generated from ImFC. This combination of ImFC and artificial intelligence can provide a more efficient and streamlined analysis compared to NGS, which often requires more complex and time-consuming data analysis. Novel developments, such as virtual freezing imaging flow cytometry, suggest that implementation of information rich cell data with artificial intelligence algorithms can further improve ImFC output and throughput [61]

In conclusion, while NGS has made significant contributions to our understanding of haematological diseases at a molecular level, ImFC offers unique advantages in terms of high-throughput capabilities, detailed morphological information, deep learning integration and label-free sorting. As such, ImFC represents a powerful tool in the study of haematological diseases and holds great promise for the future of diagnostics and research in this field.

6. Future Perspectives

ImFC is a relatively new technology in the field of cytometry. However new ImFC systems are currently under development that have increased speed and analytical power [62,63]. A stimulating example of ImFC developments is Ghost Cytometry, described by Ota et al. as an image-free rapid fluorescence "imaging" cytometry which implements a single-pixel detector of spatial information obtained from the motion of cells relative to a static optical structure. Such data computationally reconstruct cell morphology and allow rapid, accurate and inexpensive analysis [64,65], as well as the potential of cell sorting [66]. Recent advancements of Ghost Cytometry are supported by conference presentation and a recent preprint, which provide evidence on the utility of the methodology and it's application in the detection of acute leukemia cells, respectively [67,68]. Additional, larger studies and by diverse research groups will be needed to verify these promising results.

A recent development that offers unparalleled detail on phenotype analysis is Imaging mass cytometry (ImMC), a novel technology that combines flow cytometry and mass spectrometry using a laser scanning a tissue sample. The main difference to ImFC is that ImMC rather than analyzing single cells that pass through a laser in suspension, is the use of laser ablation directly to a cell surface that generate plumes of particles that are subsequently analysed with a mass cytometer [69]. ImMC offers several advantages to standard immunohistochemistry assessment, that has a limit of analysed

parameters. Antibodies that are coupled to isotopes allow for the simultaneous analysis of up to 40 parameters, with the high resolution offered by mass spectroscopy [70]. The application of ImMC allowed for multiplexed imaging of several types of samples, including normal and malignant tissues at a high resolution that reaches subcellular level [69,70]. The analytical potential of ImMC, based on both mass cytometry and histology imaging, may prove to be transformational moving towards analyses such as cancer pathology [71,72]. However, the complexity of sample preparation, the high cost and challenges in further developing ImMC technology might decelerate its application in basic and clinical research in the near future [69]. The field remains active with several recent advancements, including denoising methods [73], data analysis tools [74], intelligent tools [75] and further applications in oncology [76]. All these improvements point towards an implementation in research and clinical environments in the near future.

7. Conclusions

Imaging Flow Cytometry (ImFC) is a technology that has gained ground during the last years based on its potential in both research and clinical settings, and a promise of revolutionizing our ability to study, quantify, depict and ultimately understand cellular mechanisms. In the field of basic and translational research, ImFC provides information about to cellular morphology in addition to the high throughput capabilities of a flow cytometer, illuminating our understanding of cellular structures and their respective functions. By allowing for high-throughput analysis of individual cells in a population, it offers a detailed and objective view of cellular dynamics and enabling researchers to observe processes like cell division, antigen distribution, and intracellular pathogen behavior in real-time. The additional layer of morphological data greatly aids in the study of complex biological phenomena and can significantly enhance accuracy. On the clinical setting, even though current data are limited, the high precision of ImFC and the ability to perform label-free sorting, as well as the compatibility with machine learning algorithms indicate a promising future potential for diagnostic applications. From studying disease progression at the cellular level to potentially tailoring patient-specific treatments, ImFC might improve patient care, given that it is successfully implemented. Its capacity for real-time analysis of large cell populations also holds a promise to be implemented in high throughput clinical workflows, potentially leading to a quicker diagnosis and treatment. In conclusion, the impact of ImFC in both research and clinical environments signals a promising future for cell-based studies and medical practices.

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