**IN VIVO CELL TRACKING AND SIGNALLING WITH VARIOUS IMAGING MODALITIES**

ABSTRACT

Advancements in imaging technologies have revolutionised the field of immunology by enabling the visualisation of immune cell behaviour and signalling pathways at unprecedented spatial and temporal resolutions. This is a comprehensive review based on various imaging modalities in immunology. It explores various modern imaging techniques, including fluorescence, confocal, TIRF microscopy, SRM, TEM, AFM, etc. Each method offers unique insights into immune processes, from antigen presentation and immunological synapse formation to cell activation and migration. The integration of 3D imaging models, SMI, and multimodal approaches has further enhanced our understanding of immune cell dynamics in both in vivo and in vitro contexts. Applications extended to the study of dendritic cells, T and B cells, neutrophils, and their interactions in health and disease. Furthermore, the development of non-invasive molecular imaging modalities like MRI, PET allows real-time tracking of immune tolerance and therapeutic strategies and shaping the future of personalised immune medicine.

Keywords: Immune cell imaging, TIRF Microscopy, Flow cytometry, Immune tolerance, DNA FISH, Multimodal imaging (MRI, PET, CT, SPECT), 3D imaging

INTRODUCTION

The cell imaging techniques have enabled a better &advanced understanding of the cellular structures and functions. It has become the basis of all cellular discoveries and is playing a major role in immunology. This has helped in knowing how various immune reactions are initiated and controlled in the human body, leading to proper diagnosis and treatment of various diseases. Various imaging techniques with various resolutions and sensitivity of detection, along with the presence of a vast variety of fluorescent molecules, have greatly improved the analysis method. Immune signalling processes occur in two types – spatial and temporal scales. Spatial scale includes large micron-sized clusters to small micro and nanoclusters that are below the resolution of light microscopy, like cytokine and chemokine-based signalling, and inflammatory reactions. Etc. Temporal scale includes events ranging from microseconds to stable cell-cell interactions lasting for a few seconds to minutes. So, a single imaging technique cannot identify all these different interactions; hence, various imaging techniques with varied resolutions are used to study the cellular interactions and various biological signalling pathways. In this review, we shall discuss the various imaging techniques and their role in highlighting the various molecular mechanisms of immune cell signalling and other interactions [1].

OVERVIEW OF IMAGING TECHNIQUES

Molecular imaging techniques allow real-time observation and evaluation of immune mechanism changes in living organisms, leading to the formulation of new approaches to early disease diagnosis and adequate therapy and treatment. Different biological phenomena like the migration, differentiation, proliferation and metabolic alterations of immune cells can be traced. Another use of these methods is preclinical assessment of novel drugs on molecular targets within live cells.

Advanced microscopic methods like fluorescence and confocal microscopy visualise specific cellular proteins, structure and behaviour of immune cells in real time. Fluorescence microscopy plays an important role in studying cellular processes like antigen presentation and immune cell activation.

Two-photon microscopy, a deep tissue imaging technique, provides valuable insight into immune cell structures and their interactions within intact tissues. TIRF microscopy and scanning disk confocal microscopy provide novel insights into spatiotemporal relations b/w APCS and T-Cells. Super-resolution microscopy, including STORM and PALM, have transformed the analysis of molecular structures at the nanometre level and greatly increased our knowledge of cell membrane structure and function. Flow cytometry provides high-throughput analysis of immune populations, thus facilitating the study of various diseases. Electron microscopy, an ultrahigh-resolution imaging technique, observes the ultrastructure of immune cells and processes with very minute details that are often not captured by conventional light microscopy. In addition, the imaging techniques have revolutionised our understanding of molecular and signalling processes in T-cell activation, influencing our comprehension of immune activation and regulation [2].

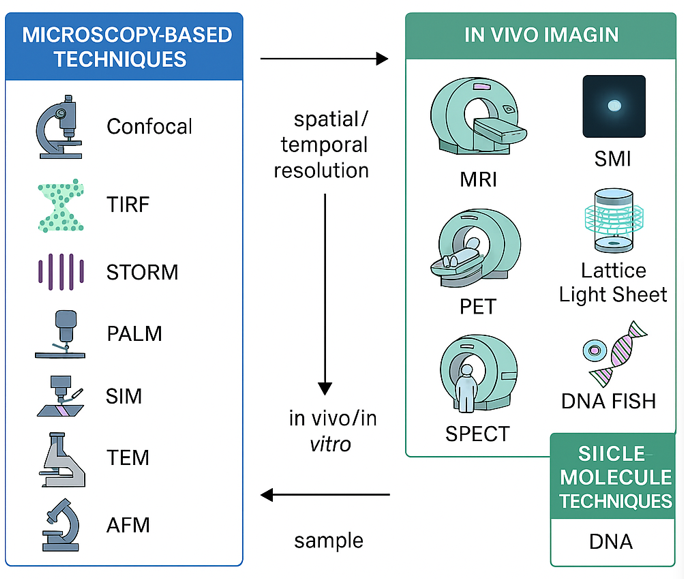


Fig 1 VARIOUS IMAGING TECHNIQUES

* 3D IMAGING METHODS AND ITS APPLICATION

Three-dimensional imaging has emerged as a very powerful tool for studying the immune system and cancer immunology. This allows for detailed examination of immune cells within their natural microenvironment, thus providing insights into cell-cell interactions and inflammatory responses. These methods, along with some other high-throughput “-omics” techniques, develop some mechanistic models that describe immune system behaviour across multiple scales. In oncology-related studies, 3d in vitro models such as organoids, microfluidic cultures, and bioprinting are more advantageous over traditional 2D models by providing a better understanding of tumour replication and allowing co-culture of immune cells [3].

Table 1 3D IMAGING MODELS

|  |  |  |  |
| --- | --- | --- | --- |
| MODEL TYPE | APPLICATION | IMAGING METHOD | ADVANTAGE |
| Organoids | Tumor immune interaction | Confocal, LLSM | Mimics in vivo environment |
| Microfluidic chips | Immune migration | TIRF, SRM | Real-time perfusion |
| Bioprinted constructs | Immunotherapy testing | Multi-photon, AFM | 3D architecture & functioning |

* + 3D IMAGING IN IMMUNE SYNAPSE STUDY

Fluorescence images of an immunological synapse were initially captured with confocal microscopy [4][5]. Earlier, most immune signalling studies relied on Commercial spinning disk confocal microscopes with diffraction-limited resolution (lateral: 200 nm, axial: 500 nm) with video rate 2D imaging (20 Hz), examples such as release of cell killing particles [6], or calcium influx [7]. The main disadvantage of this method is limited speed and high chances of photobleaching. However, this has been avoided in 3D imaging, which can capture structures like the cell-cell interface.

Widefield fluorescence methods use cameras to transcend the shortcomings of confocal microscopy, albeit with less axial sectioning. Structured Illumination microscopy (SIM) is a super-resolution microscopy method that employs interface patterns to generate a fluorescence image from different directions. A combination of SIM with new techniques, such as image correlation spectroscopy, has made membrane protein dynamics analysis faster [9].

Lattice light-sheet microscopy (LLSM) provides high-speed and high-resolution imaging with minimum phototoxicity and photo bleaching effects. This method has been the most promising technique for immune cell 3D imaging, such as unveiling the topological changes of T cell IS formation[10], how finger-like cellular protrusions search for antigens[11] and how the actin cytoskeleton supports immune activation [12][13].

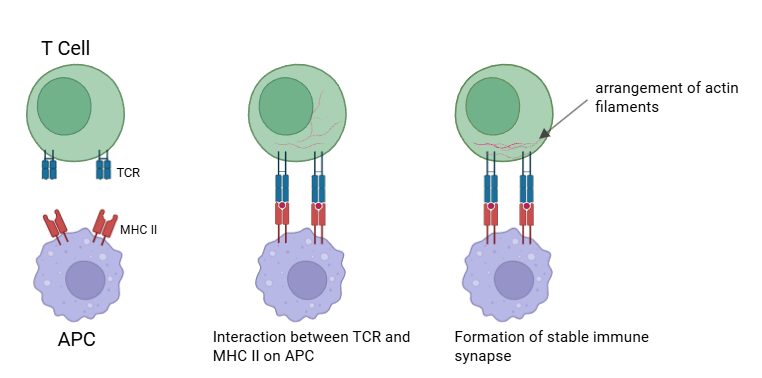
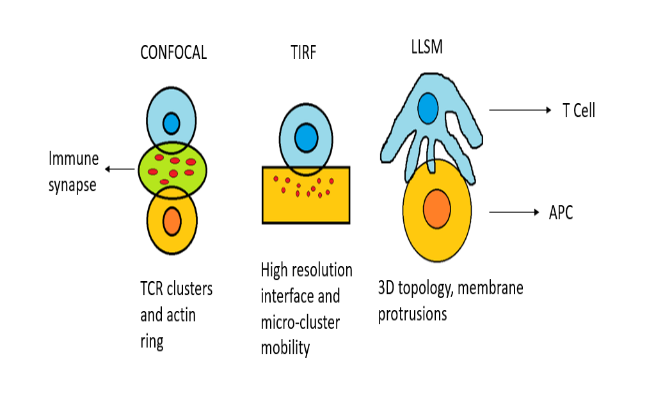
 

Fig 3Immune synapse visualisation using various microscopy techniques

Fig 2 Formation of Immune synapse

* IN SITU SINGLE MOLECULE IMAGING OF IMMUNE CELL

Recent advancements in single-molecule imaging techniques have helped revolutionise our knowledge and understanding of immune cell functions and behaviour [14][15]. Single-molecule level in situ imaging of the cell membrane provides crucial insights into molecular structures and signal transduction [16][17]. This requires high sensitivity, fast acquisition and minimal photobleaching. T cell receptor signalling, immune synapse formation are investigated using SMI[18].Single-cell imaging based on total internal reflection fluorescence microscopy at the basal cell interface is widely employed to investigate the initiation of immune response by the T cell receptor. Single-molecule light-sheet microscopy allows imaging of single receptors in any plane within a cell to investigate protein dynamics and organisation within suspended T cells. The light sheet generated high-quality single-molecule fluorescence images that could be matched to those acquired by total internal reflection fluorescence microscopy, thereby elucidating how protein diffusion and cellular activation are influenced by surface contact [19].

In studying granulomas in tuberculosis, Single-molecule fluorescent in situ hybridisation was used for developing multiplex imaging of mRNA markers, enabling the classification of immune cell subtypes and their distribution in the granulomas[20]. Intravital microscopy enables real-time observation of immune cell behaviour in living tissues, their migration pattern, cell-cell communication and regulation[21]. These techniques collectively aid in investigating immune cell dynamics at unprecedented resolution, advancing our understanding of immunology and potential therapeutic interventions.

* HOW ELECTRON MICROSCOPY REVEAL PREVIOUSLY UNDETECTED MORPHOLOGICAL VARIATIONS IN NEUTROPHIL ACTIVATION DURING INFLAMMATORY PROCESSES

Electron microscopy has helped in revealing significant morphological variations in neutrophil activation during inflammatory processes. Visualisation of neutrophils by transmission electron microscopy(TEM) explained the morphology of the para-inflammatory phenotype of oral neutrophils & comparing it with the naïve blood neutrophil, revealed that proinflammatory neutrophils have fewer granules, lighter cytoplasm and greater nuclear euchromatin compared to para-inflammatory neutrophils[22]. Activated neutrophils develop more prominent cytoplasmic structures and processes[23]. Confocal and fluorescence microscopy, although employed to examine neutrophil activation and NET-osis, do not expose the neutrophil membrane surface, its nanostructure, and morphology. Atomic force microscopy can reveal the changes in neutrophil membrane structure at the nanoscale level during activation and NET-osis, including cell spreading, fragmentation, and membrane disruption[24].TEM revealed the development of vacuoles in electropermeabilized neutrophils, upon stimulation, along with the fusion of azurophilic and specific granules in both the vacuoles as well as cell membrane[25].

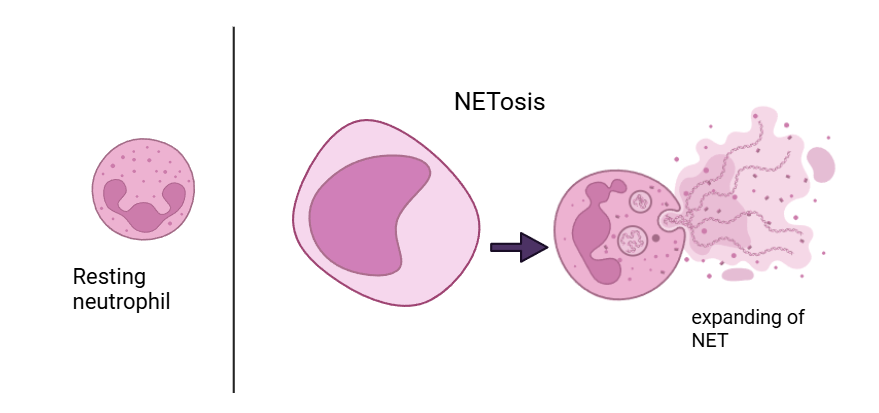


Fig 4 Visualisation of neutrophils during NETosis using electron microscope

* CELL IMAGING TECHNIQUES IN UNDERSTANDING THE FUNCTIONING OF DENDRITIC CELLS

Dendritic cells are the primary antigen-presenting cells (APC) and link innate and adaptive immunity. Advances in imaging methods focusing on live cell imaging in situ have opened up new windows of understanding the interactions between antigen-presenting cells and T cells. Techniques such as TPM, TIRF microscopy, and SDCM allowed high-resolution imaging and deep visualisation into dendritic cell processes in real time[26]. Advanced imaging methods and high resolution illuminating instruments like intravital imaging by multiphoton microscopy, LSM, AFM, and SRM allowed us to determine the specific cell subtypes, monitor the cell migration with time using time-lapse imaging, observing the in vivo cell contacts in the resolution of individual cells, and investigating the molecular mechanism responsible for the cell behavior and interaction, like the intravital imaging monitors dynamic organisms within living, intact animals to generate continuous and simultaneous information in an intact organism with full interactions. Atomic force microscopy provides high-resolution nanoscale images of the cell surface. STORM and STED are the most widely used super-resolution methods, which are employed to expose the nano-level structure of various structures in cells. These techniques enabled researchers to observe dendritic cell manufacturing, antigen capture and presentation, as well as their migration and interaction with T cells during any immune response[27]. Spatiotemporal tracking of dendritic cells has revealed their role during the period of infection and inflammation, giving important insights into their role in initiating an adaptive immune response.[28]. Moreover, recent developments in multiplexed imaging techniques have enabled the study of the spatial localisation of dendritic cells within tissues, providing valuable information about cellular cross-talk and tissue-specific functions[29]. These advancements have significantly enhanced our understanding of dendritic cells and their role in immune regulation.

* CONFOCAL MICROSCOPY IMAGING IN T-CELL AND B-CELL ANALYSIS

Advanced confocal microscopy has greatly revolutionised our understanding of lymphocyte dynamics and the study of T and B cells. These have offered high spatial and temporal resolution, thus enabling direct observation of cellular activation processes. Electron and sophisticated light microscopy methods have been utilised to generate high-resolution images of lymphocytes in vitro. High-resolution techniques have overcome the diffraction limit of light to examine subcellular characteristics as minute as a single molecule. Advances in imaging technologies have therefore allowed visualisation of signalling events in lymphocytes with increasingly higher spatial and temporal resolution.[30]. Super-resolution microscopy methods, such as Airy-Scan, STED, and TEM, have improved imaging of mitochondrial morphology and metabolism in B and T cells, demonstrating activation-induced modifications and internal structural modifications[31].Moreover, recent imaging modalities like TPM, TIRF microscopy, and SDCM have confronted current dogma and offered new knowledge regarding dendritic cell function and spatiotemporal interactions between antigen-presenting cells and T cells. These imaging methods together provide unparalleled cellular and molecular resolution, supporting the validation, revision, and replacement of lymphocyte activation models.

* SUPER RESOLUTION IMAGING IN STUDYING IMMUNE SIGNALLING PATHWAYS

Super resolution microscopy has revolutionised our knowledge of immune cell signalling by visualisation at the nano scale, protein organisation and dynamics. This method has been used for studying immunological synapses, revealing valuable details about receptor distribution and signalling processes. Super-resolution techniques have provided new insights into NK Cell biology. Recent developments, such as SPARCOM with improved resolution, enabled real-time imaging of T Cell receptors during the activation process[32]. Several super-resolution techniques, such as 3D-SIM[33], STED[34], SMLM, TIRF[35] and LLSM[36] [37] have been used for the visualisation of actin dynamics and lytic granules within the immunological synapse of T Cells and NK Cells. DNA FISH is used to quantify the nuclear position of the immunoglobulin heavy chain locus on B-cell progenitors.[38]

Techniques like STORM[39], PALM[40][41], PAINT[42] or GSDM[43]cause the fluorophores to switch between visible and invisible states [44]. Conditions are chosen as such that most molecules become invisible and hence do not get imaged. Thus, the average distance b/w visible molecules becomes greater than the resolution limit of the microscope, and individual molecules can now be localised. By recording several such images, a super-resolution image can be produced. These are often used for the determination ofthe local and global distribution of molecules on the cell membrane.

These advanced imaging techniques have answered long-standing immunological conundrums and are still providing promising directions for future work in molecular immunity. The use of super-resolution microscopy to image immune cells has greatly improved our comprehension of intricate immunological processes and their spatial organisation [45].

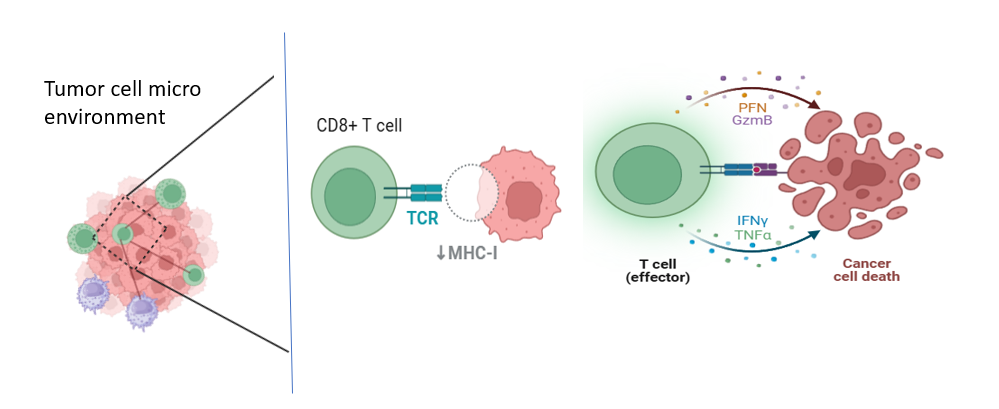


Fig 5 Visualisation of T-Cell interaction with tumor cell at tumor micro environment with super resolution imaging techniques

* ADVANCED MOLECULAR IMAGING TECHNIQUES ALLOWS REAL-TIME VISUALISATION OF IMMUNE CELL INTERACTIONS DURING IMMUNE TOLERANCE

Immunologic tolerance and immune metabolism are essential to immune homeostasis and the immune response against disease. Advances in molecular imaging technologies, particularly optical molecular imaging, nuclear medicine imaging, and magnetic resonance imaging, have led to a tremendous improvement in imaging immune tolerance and immune metabolism. The technologies of molecular imaging allow for real-time monitoring and analysis of dynamic immune tolerance processes and immune metabolism in living beings, enabling to development of novel strategies for early diagnosis of disease, targeted therapy, and immunotherapy [46]. These techniques allow non-invasive tracking of immune cells in vivo, thus providing better visualisation of cellular behaviour and therapeutic responses[47][48]. Multiple imaging modalities, such as MRI, CT, PET, SPECT, and optical imaging, possess distinct strengths for immune cell tracking[49]. Multimodal imaging strategies leverage the strengths of each method to surmount its weaknesses. These technologies have enabled the creation of cell-based therapies, like NK cell-based cancer immunotherapies. Molecular imaging has improved our knowledge of immune priming, tolerance, and T-B cell interactions. Nonetheless, scientists need to take into account possible physiological effects when developing immune-monitoring imaging tracers. In general, these advances in imaging technologies are essential for maximising immune cell therapies and understanding intricate immunological mechanisms [50].

Table 2 COMPARISON OF IMAGING TECHNIQUES IN IMMUNE CELL STUDIES

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| TECHNIQUES | RESOLUTION | SAMPLE TYPE | STRENGTH | LIMITATIONS |
| Confocal | ~200 nm | Live/fixed cell | 3D imaging | Limited depth |
| TIRF | ~100 nm axial | Cell membrane | Surface interaction | Only surface imaging |
| STORM | ~20-30 nm | Fixed cell | Nanoscale resolution | Complex setup |
| MRI | ~100 um | In vivo | Non-invasive, deep tissue | Low molecular resolution |
| PET | ~1-2 mm | In vivo | Functional tracking | Radiation exposure |

Table 3 APPLICATION OF IMAGING TECHNIQUES TO IMMUNE CELLS

|  |  |  |
| --- | --- | --- |
| IMAGING TECHNIQUE | CELL TYPE STUDIED | KEY INSIGHTS |
| LLSM | T cells | Topological changes in IS formation |
| Electron microscopy | Neutrophils | Membrane granule morphology |
| Multiphoton microscopy | Dendritic cell | Intravital dynamics |
| DNA FISH | B cell progenitors | Gene locus localisation |
| Flow cytometry | Mixed | Cell population phenotyping |

CONCLUSION

Recent innovations in imaging technologies have revolutionised our knowledge of immune cell function and signalling pathways. The convergence of a variety of imaging modalities—from confocal and two-photon microscopy to super-resolution approaches such as STORM, PALM, STED, and lattice light-sheet microscopy—has been achieved to visualise immune events at the cellular and molecular levels with great accuracy. These methods have enabled real-time observation of dynamic processes such as immune synapse formation, antigen presentation, activation of T and B cells, and dendritic cell movement. Moreover, high-throughput and ultrastructural approaches like flow cytometry, TEM, and AFM have extended our assessment capabilities further, providing detailed insights into immune cell morphology, heterogeneity of populations, and subcellular structure. The use of these technologies has not only added substantially to fundamental immunological research but also has provided insight into the development of therapeutic approaches, most notably in cancer immunotherapy, infectious disease surveillance, and autoimmune diagnostics.

FUTURE ASPECTS

In the future, immune imaging will facilitate real-time imaging of complex biological processes in their native physiological context with faster, more sensitive, and less invasive methods. The need is acute for modalities with improved spatial and temporal resolution without phototoxicity, especially for live-cell and in vivo imaging. The combination of artificial intelligence and machine learning with imaging platforms holds the promise of transforming image analysis by facilitating pattern recognition, predictive modelling, and large-scale interpretation of data in an automated way. Additionally, Innovations such as machine learning-integrated image analysis and high-content screening technologies will speed up the visualisation of dynamic immune processes with unprecedented resolution. The development of patient-derived 3D models such as organoids, microfluidic systems, and bio-printed immune tissues will greatly increase the physiological relevance of experimental systems, especially in cancer immunology and personalised medicine. Moreover, the integration of multiplexed and multimodal imaging strategies—combining optical methods with MRI, PET, SPECT, and molecular probes—will yield comprehensive insights into immune dynamics at cellular, tissue, and systemic levels. These advances will further our mechanistic insight into the immune system, inform the development and assessment of future immunotherapies, and facilitate the application of precision medicine. As these technologies continue to become more precise, scalable, and accessible, they are likely to become essential tools in research and clinical settings, determining the future landscape of immunological discovery and therapy.

ABBRIVIATIONS

STORM-Stochastic Optical Reconstruction Microscopy

PALM-Photo Activated Localisation Microscopy

SIM-Structured Illumination Microscopy

LLSM-Lattice Light Sheet Microscopy

SMI-Single Molecule Imaging

TEM-Transmission Electron Microscopy

TPM-Two-Photon Microscopy

TIRF-Total Internal Reflection Fluorescence Microscopy

SDCM-Scanning Disc Confocal Microscopy

LSM-Light Sheet Microscopy

AFM-Atomic Force Microscopy

SRM-Super Resolution Microscopy

STED-Stimulation Emission Depletion Microscopy

GSDM-Ground State Depletion Microscopy

SMLM- Single Molecule Localisation Microscopy

PAINT-Point Accumulation for Imaging in Nanoscale Topography

SPECT-Single Photon Emission Computed Tomography

PET-Positron Emission Tomography

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