

Metal Isotope-Tagged Nanomaterials for Mass Cytometry and Imaging: Advances and Biomedical Applications

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Abstract

Mass cytometry (cytometry by time-of-flight (CyTOF)) and imaging mass cytometry (IMC) are transformative technologies that combine flow cytometry principles with time-of-flight mass spectrometry (TOF-MS). By employing metal isotope-tagged antibodies instead of fluorophores, these techniques overcome spectral overlap limitations and enable high-dimensional, compensation-free analysis of complex biological systems at single-cell resolution. The performance of CyTOF and IMC critically depends on advanced nanomaterials labeled with stable metal isotopes, which are essential for improving sensitivity and multiplexing capacity. This review systematically discusses the design principles, synthesis methods, and functionalization strategies of mass-tagged nanomaterials tailored for CyTOF (e.g., cell suspension analysis) and IMC (e.g., spatial proteomics of tissue sections). We highlight their impactful applications in biomedicine, including proteomics, immunology, oncology, and neuroscience, emphasizing their roles in disease diagnosis, targeted drug development, and single-cell analysis. Despite these advancements, challenges such as nanomaterial biocompatibility, clinical scalability, and artificial intelligence (AI)-driven design are discussed, providing a roadmap for future research in personalized medicine and theranostics.

Keywords: mass tags; mass cytometry; imaging mass cytometry; proteomics; biomedical fields

Introduction

With the ongoing advancement of life sciences, high-throughput, multiparameter single-cell analysis technologies have become central to research.

Traditional flow cytometry plays a vital role in multiparameter detection but faces significant limitations in high-parameter multiplexed labeling and spatial information acquisition. These include limited parameter throughput (typically fewer than 20

markers), difficulty in resolving complex cell subpopulations, a narrow dynamic range of fluorescent signals (approximately 3–4 orders of magnitude), insufficient sensitivity for low-abundance antigens (<100 molecules per cell), and the need for complex spectral compensation—all of which can compromise data reliability if compensation errors exceed 15% [1].

To address these issues, metal isotope-labeled mass cytometry was developed [2, 3]. CyTOF combines the advantages of flow cytometry and mass spectrometry by replacing traditional fluorescent dyes with metal isotope labels (often called “mass tags”). As illustrated in Fig. 1, a schematic diagram depicts the working principle of CyTOF. Coupled with an inductively coupled plasma time-of-flight mass spectrometer (ICP-TOF-MS), this technology allows simultaneous detection of over 100 stable heavy metal isotopes. Because each metal has a unique mass-to-charge ratio (m/z), CyTOF circumvents the channel cross-interference inherent to fluorescence-based methods, enabling high-throughput, multiplexed, and highly precise analyses [4]. The number of detectable marker channels can exceed 40, with sensitivity reaching approximately 10 molecules per cell, facilitating the detection of low-abundance signaling molecules [5, 6]. Additionally, the exceptional stability of metal tags significantly enhances the

dimensionality of cell phenotyping and functional analysis. This technology has been widely applied in areas such as signal transduction, tumor microenvironment (TME), autoimmune diseases, infections, immunotherapy, and organ transplantation, underscoring its broad potential for future application [4, 7, 8].

Beyond single-cell non-spatial analysis, the need for spatial, tissue-specific insights has catalyzed the development of imaging mass cytometry (IMC). Derived from CyTOF, IMC employs laser ablation to scan tissue sections, releasing ions from metal-tagged antibodies, which are then detected via mass spectrometry to visualize protein distribution in situ [9]. Figure 2 presents a schematic of the IMC workflow. This technique preserves spatial architecture (resolving microenvironments of fewer than 10 cells), integrates morphological and proteomic data (compatible with H&E staining and 40-marker multiplexing), and enables detailed characterization of cell interactions and microenvironmental features in the spatial context [10]. IMC has been widely applied in studies of tumor immune microenvironment, neural tissues, and organ localization research [11, 12].

Mass tags (or metal tags) have been central to the evolution of metal isotope labeling technology, with their innovative design significantly advancing high-

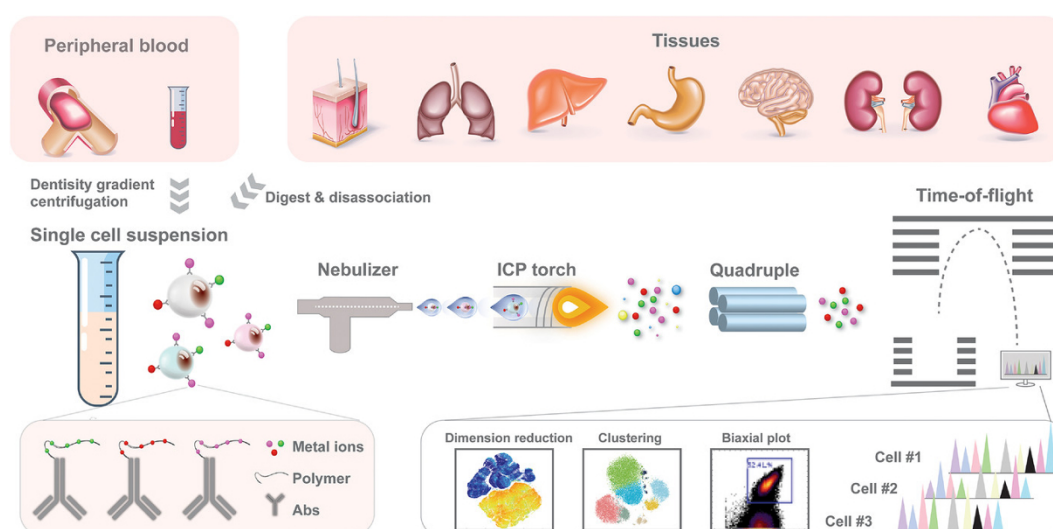


Fig. 1 Schematic diagram of CyTOF working principles. Target antigens are labeled with antibody-mass-tag conjugates. Once labeled, the cells are introduced into a plasma torch, where they undergo atomization and ionization, resulting in the formation of an ion cloud. Subsequently, a quadrupole filters out low m/z ions allowing only heavy isotope ions to pass through. These ions are then detected by a time-of-flight detector, which provides single m/z resolution. The signal intensity measured by CyTOF directly correlates with the number of target epitopes present per cell, enabling precise biomolecular profiling. Reproduced from Ref. [6] © 2020 The Authors. Clinical and Translational Medicine is published by John Wiley & Sons Australia, Ltd. on behalf of Shanghai Institute of Clinical Bioinformatics.

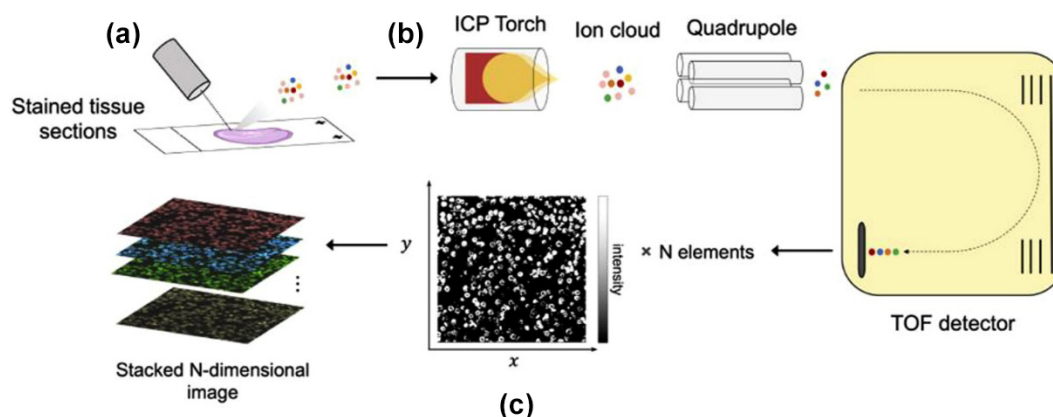


Fig. 2 the schematic workflow of IMC. **(a)** Tissue sections are initially stained with antibody-mass-tag conjugates and mounted in a coverslip chamber. A UV laser rasterizes the sample into $1\ \mu\text{m}^2$ pixels, producing an aerosolized cloud of tissue particles. These particles are carried into the inductively coupled plasma (ICP) torch by an inert gas flow. **(b)** The CyTOF detection system measures the isotope content of each pixel, enabling detailed analysis. The final output is a 2D image in which signal intensity reflects the concentration of each target epitope, providing valuable insights into the spatial distribution of biomolecules within the tissue. Reproduced with permission from Ref. [4] © 2023 the American Chemical Society.

throughput and multiplex analysis in mass cytometry. Early studies employed inductively coupled plasma mass spectrometry (ICP-MS) to monitor protein antigens, primarily for bulk sample detection, laying the groundwork for subsequent multiplex labeling techniques [13]. In 2006, the concept of using multiple distinct isotope tags at the cellular level was proposed, prompting research to incorporate commercially available rare earth metals (e.g., samarium (Sm), europium (Eu), and terbium (Tb)) for multiplex quantitative analysis of cellular antigens [14]. Initially, antigen detection relied on dissociation-enhanced lanthanide fluorescence immunoassay reagents, containing 6-9 copies of naturally abundant lanthanide elements. This limitation resulted in lower sensitivity, restricting detection to highly expressed biomarkers. To address this, multiplex metal-chelating polymers (MCPs), such as polyacrylamide copolymers incorporating side-chain N, N-dimethylamino groups and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelators, have been developed. These polymers significantly improve detection sensitivity by multivalently binding metal ions to antibodies, expanding detection range and establishing a solid foundation for multiplex metal tags [15]. The primary advantage of metal isotope tags lies in their unique m/z values, enabling highly multiplexed analysis to precisely track numerous biomarkers in single cells across spatial and temporal scales. This innovation enhances the resolution of cellular phenotypes and functions, facilitating the systematic construction of high-dimensional cellular phenotype landscapes

across diverse biological contexts. These advancements not only advance fundamental biological research but also open new frontiers in clinical diagnosis and personalized medicine.

This review systematically discusses the design of metal labels, labeling strategies, and key technical parameters in CyTOF and IMC, evaluates current applications and progress in immunology, oncology, neuroscience, and other fields, analyzes existing technical challenges, and anticipates future development trends. The aim is to provide a theoretical foundation and a technological roadmap to promote the clinical translation of mass spectrometry-based cellular analysis and precision medicine.

Types of Metal Tags and their Evolution

Metal isotope tags are chemical or isotopic moieties used to label biomolecules (e.g., proteins, nucleic acids, metabolites) to enhance detection, quantification, and multiplex analysis in MS-based workflows. By introducing distinct mass signatures or chemical reactivities, these labels address key challenges in biomolecular profiling, such as low abundance, spectral overlap, and sample throughput [4].

These labels are broadly classified into four generations, each representing a significant technological advancement. First-generation metal isotope tags developed in 2006 for CyTOF, utilize

stable metal isotopes such as lanthanides and platinum for multiplexed single-cell analysis. The unique m/z signatures enable detection of up to 100 markers simultaneously, overcoming spectral crosstalk limitations of fluorescent dyes. Second-generation tags employ chelating backbones (e.g., dextran, dendrimers) to facilitate multivalent metal loading, allowing tens to hundreds of ions per antibody. This significantly increases signal intensity for low-abundance antigens and enhance stability in complex biological matrices. Third-generation nanoparticle-based tags leverage optical and electronic properties of materials, such as mesoporous silica nanoparticles (MSNs) and nanoscale metal-organic frameworks (NMOFs) for signal amplification and targeted delivery. For example, antibody-conjugated gold nanoparticles enable both mass spectrometric detection and tumor-targeted imaging in preclinical models. Finally, bead-based barcodes and cell-indexing tags encode cell identities via DNA-based indices or barcoded microparticles for high-throughput single-cell analysis. They encode cell identities with barcoded microparticles or DNA indices, enabling pooling of thousands of cells per assay and significantly reducing reagent costs and analysis time.

Metal isotope tags

The metal isotopes used in mass cytometry fall into two categories: (i) lanthanides (excluding radioactive promethium (Pm)), such as lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), samarium (Sm), europium (Eu), gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb), and lutetium (Lu)); and (ii) high- Z metals ($Z \geq 74$), such as platinum (Pt), palladium (Pd), iridium (Ir), gold (Au), tungsten (W), bismuth (Bi), osmium (Os). These metals offer advantages as mass tags due to their distinct m/z , minimal spectral interference, and high stability. Lanthanides are favored because their electronic configurations, particularly the stable 4f orbitals, facilitate strong chelation and minimal spectral interference. High- Z metals ($Z \geq 74$), such as Pt, W, Bi, etc., provide benefits like distinct mass signatures separated from endogenous biological elements (C, N, O, Na, K, and Ca), making them suitable for ultra-high-parameter cytometry and multimodal imaging. Non-metal iodine (I, 127 Da) can also serve as an antibody tag, detected by ICP-

MS, broadening labeling options for molecules lacking metal-binding sites.

Selection favors isotopes that are biocompatible and produce minimal background; ideal isotopes are non-toxic, non-radioactive, and absent or trace in biological systems. Han et al. conjugated monoclonal IgG antibodies with 48 metal isotopes, including 14 lanthanides and 6 palladium isotopes, using bifunctional chelators like DOTA, ensuring stable binding through site-specific reactions. They also developed a lyophilization-based palladium tag preparation for immunophenotyping [16]. H. Fienberg et al. developed a platinum-based reagent for live-cell analysis, addressing the incompatibility of fluorescent dyes with mass cytometry [17]. Using cisplatin, they discriminated live/dead cell based on platinum signals from DNA-crosslinked dead cells. Unlike fluorescent dyes, which suffer from spectral overlap, this method allows interference-free quantification within one minute, with results comparable to fluorescence assays but without DNA damage under short-term treatment (≤ 6 h). Li, et al. proposed a platinum-chimeric carrier cell approach to address the need for large cell numbers in CyTOF [18]. Labeling carrier cells with cisplatin amplified signals from rare populations (e.g., 10 000 immune cells), reducing sample volume requirements. This approach helps overcome challenges of scarce clinical samples such as circulating tumor cells (CTCs) in early cancer diagnosis and minimal-invasive pediatric biopsies, where limited sample volume hinders analysis. High- Z metals like platinum are pivotal in translational research, enabling sensitive detection in limited samples.

Metal chelate polymer tags

Stable labeling of antibodies with metal isotopes via chelators is essential for enabling mass spectrometry detection. Early metal labeling methods, which relied on small molecule chelators such as DOTA and EDTA, exhibited several limitations. First, each chelator could bind only one metal ion, restricting the metal loading per antibody and reducing sensitivity for low-abundance antigens. Second, direct conjugation often disrupted antibody structure, impairing antigen-binding capacity. Third, small molecules are susceptible to metal ion dissociation in complex biological environments, increasing background noise [19].

To address these issues, metal chelate polymer (MCPs) have emerged as a promising solution. MCPs feature multivalent chelation through polymer backbones such as dextran or dendrimers. Allowing a single polymer chain to bind tens or hundreds of metal ions, this significantly enhances signal intensity. Compared to conventional methods requiring high antibody concentrations, polymer-based labeling can utilize high-affinity, kinetically inert chelators (e.g., DTPA derivatives) that confer stable metal binding under acidic conditions and during prolonged storage, thereby maintaining signal integrity in mass cytometry.

In response to the dual needs of fluorescence imaging and mass cytometry, Wu et al. reported a novel lanthanide-coordinated semiconductor polymer dot (Pdot) probe [20]. This probe integrates the intrinsic fluorescence of Pdots with lanthanide-based mass signals. They prepared Pdot-PC-Ln-COOH by chelating lanthanide ions with carboxyl-functionalized polymer PC, followed by mixing with PS-PEG-COOH block copolymer. The probe exhibits strong fluorescence, making it suitable for flow cytometry, and surpasses commercial mass cytometry probes in sensitivity and multiplexing capacity. By adjusting the Pdot size and the lanthanide elements, a series of probes were designed for multiplexed single-cell analysis. Illy et al. synthesized a novel MCP via anionic ring-opening polymerization of activated cyclopropane (1,1-cyclopropane dicarboxylic acid diallyl ester), modified with 2-furoyl methanesulfonic acid and phosphine oxide [21]. This controlled polymerization produced polymers with predictable molecular weights, narrow dispersity, reactive side groups for metal chelator attachment, and orthogonal end groups for monoclonal antibody (mAb) conjugation. Post ring-opening, diethylenetriaminepentaacetic acid (DTPA) chelators were introduced through a two-step process. The resulting polymers were characterized by nuclear magnetic resonance (NMR), size-exclusion chromatography (SEC), and thermogravimetric analysis (TGA), with Gd^{3+} -binding validated by isothermal titration calorimetry (ITC). Polymers loaded with ten different lanthanide ions and conjugated to ten mAbs enabled simultaneous 10-plex detection of cell surface antigens in human peripheral blood single-cell analysis. Lou et al. developed an elemental labeling method for multiplexed protein detection in cells using ICP-MS [15]. This approach

employed water-soluble polymers bearing multiple metal-chelating ligands. The terminal maleimide groups of the polymers conjugated to cysteine-SH groups on the Fc-region of antibodies, forming high-affinity complexes with lanthanide ions. When applied to analyze antigen expression in acute leukemia cell lines, the labeled antibodies showed no interchannel interference, enabling quantification of cell surface markers of varying abundance levels in a single assay. This approach bridges the gap between single-cell analysis and high-throughput screening, laying the foundation for large-scale cell line “fingerprinting”.

Currently, MCP's development primarily focuses on lanthanides and bismuth due to their chelation stability. For other metals such as zirconium, rhenium, and platinum, modifications to chelators and polymers are necessary. Zirconium has four isotopes suitable for mass cytometry; however, traditional aminocarboxylate chelators exhibit weak binding affinity to zirconium ions at room temperature. To address this, researchers combined MCPs with desferrioxamine (DFO) side groups via SCN-Bn-DFO and incorporated PEG side chains to enhance water solubility [22]. This polymer, containing fluorescein and biotin, effectively chelated zirconium, as confirmed through streptavidin-bead incubation and IMC. Zhang et al. developed MCPs with 2,2'-dipyridylamide (DPA) side groups for transition metals such as rhenium and platinum [23]. They employed two coupling strategies: thiol-maleimide reaction for rhenium and DBCO-azide “click” chemistry for platinum, designed to prevent interactions between platinum and thiol groups. Although DPA-based MCPs show promise for platinum labeling, nonspecific binding remains a significant obstacle. Wong et al. investigated Pt-DPA MCPs and reported nonspecific binding issues [24]. To mitigate this, they incorporated hydrophilic oligomers, such as methoxy polyethylene glycol (mPEG) and poly (sulfobetaine methacrylate) (PSBMA), to shield the polymer surface via steric and charge repulsion, offering valuable insights for developing platinum-based MCPs.

Additionally, new MCP-related polymers, such as HyperMAPs and HaloTag ligands, have been developed. Verhoeff et al. synthesized HyperMAPs, peptide-based hyper loaded metal complexed polymers with precise metal assembly achieved via

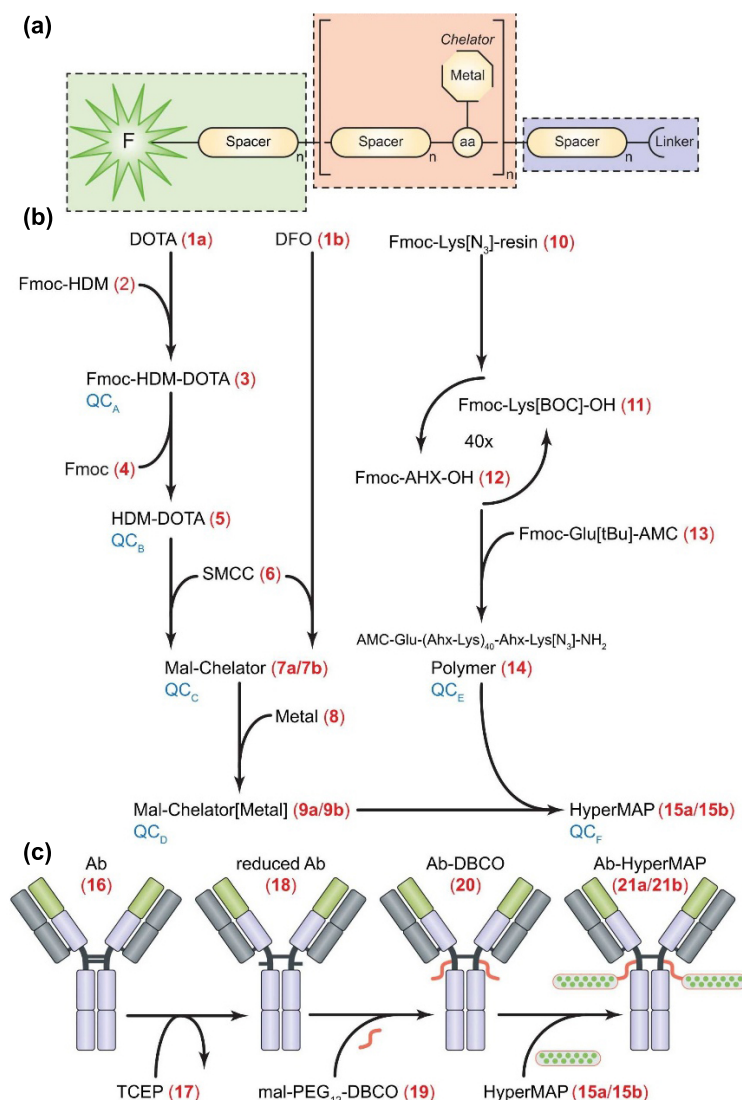


Fig. 3 Design and synthesis strategies of HyperMAPs. **(a)** Schematic illustration of the general structure of HyperMAPs. **(b)** Schematic depiction of the chemical strategy for synthesizing 15a/15b. **(c)** Schematic of HyperMAP conjugation to a monoclonal antibody. Reproduced from Ref. [25] © 2024 The Authors.

controlled solid-phase synthesis (Fig. 3) [25]. These peptide scaffolds include antibody conjugation handles, fluorescent tracking groups, and metal chelate sites. HyperMAPs can coordinate 21 metal isotopes using two chelators and demonstrated excellent performance in a 13-parameter CyTOF panel, outperforming monoisotopic metal-conjugated antibodies. Compared to conventional antibody-metal tags, HyperMAPs substantially increase metal loading. Potter, et al. designed the first HaloTag ligand for mass cytometry by conjugating “click”-reactive HaloTag warheads to poly(L-lysine)/polyacrylic acid polymers functionalized with DTPA chelates [26]. Kinetic analysis showed that the 1-chlorohexyl warhead structure influenced the labeling rate, while PEGylated reduced nonspecific

binding, enabling discrimination between cells expressing the HaloTag protein and those that do not. These findings provide a blueprint for integrating genetic tagging with mass cytometry.

In conclusion, MCPs have emerged as a transformative tool in biomedical analysis. However, challenges remain, including issues related to polymer biodegradability and the need to optimize compatibility with a broader range of metal isotopes, presenting opportunities for future research and development.

Nanoparticle-based tags

Nanoparticle-based tags have emerged as a powerful solution to address the sensitivity limitations of traditional MCP tags, particularly for detecting ultra-

low-abundance biomarkers [27]. Their effectiveness derives from high metal ion payloads (thousands of ions per nanoparticle), tunable surface chemistries, and multimodal imaging capabilities. These features enable signal amplification, reduction of nonspecific binding, and integrated biological analysis, thereby significantly enhancing detection sensitivity.

For example, Zhang et al. introduced lanthanide MOF-flowers as innovative materials for protein enrichment in ultra-trace human biofluids [28]. The sample preparation workflow combines protein capture, digestion, and peptide elution within a single PCR tube, minimizing sample loss. Using this method, approximately 3,900 proteins were quantified from 1 μ L of aqueous humor from patients with cataract and retinal vein occlusion (RVO). Machine learning identified PFKL as a significant RVO biomarker, achieving an area under the curve of 0.95 ± 0.04 , thereby enhancing biomolecular profiling and therapeutic target exploration. Li et al. designed functionalized mesoporous silica nanoparticles (MSNs) as mass cytometry probes, modified with zwitterionic sulfobetaine silane and long-chain polyethylene glycol silane (PEG_{5k}, $M = 5000$) (Fig. 4) [29]. These MSNs exhibit uniform size, excellent redispersibility, and colloidal stability, accommodating 13 lanthanides with up to 7.4×10^4 Tb ions per nanoparticle while maintaining negligible ion loss in biological buffers. Tuning the PEG_{5k} surface density effectively reduced nonspecific binding to human serum albumin and peripheral

blood mononuclear cells (PBMCs). In 10-plex PBMC staining, unbound MSNs demonstrated compatibility with commercial MCPs. Optimized N₃-terminated MSNs (PMSN-Zwi-PEG_{5k}-N₃), conjugated with anti-biotin antibodies, effectively bound biotin-Cy5, with eight bioconjugation conditions tested to enhance coupling efficiency. These findings highlight the significant potential of MSNs for mass cytometry applications.

Lin et al. further validated nanoparticle sensitivity using 700 nm aqueous microgels as cell mimics [30]. Compared to MCPs containing 50 Tb³⁺ ions per probe, biotinylated NaHoF₄ nanoparticles with 15 000 Ho atoms per probe demonstrated detection of 100–500 proteins per microgel, producing signals two orders of magnitude stronger. However, sensitivity was limited by nonspecific binding. This research illustrates that nanoparticle reagents can enhance detection sensitivity in single-cell mass cytometry, with potential to improve diagnostic capabilities in clinical settings. In another study, Pichaandi et al. developed PEG-modified silica-coated NaHoF₄ nanoparticle-based antibody mass tags for mass cytometry [31]. While these tags offered no advantage for high-abundance biomarkers, they significantly improved detection of low-abundance. When combined with MCP-antibody conjugates, they minimized nonspecific binding and increased signals from rare CD14⁺ monocyte by 20-fold in PBMCs, complementing MCP reagents for detecting rare biomarkers.

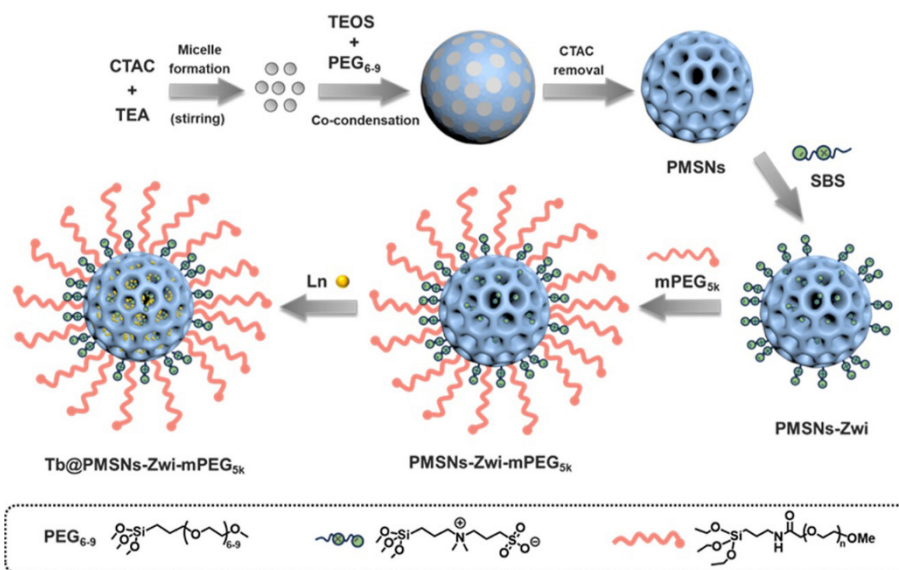


Fig. 4 Schematic diagram of the procedures for preparing multifunctional MSNs. Reproduced with permission from Ref. [29] © 2024 American Chemical Society.

Dual-modal nanoparticles, capable of combining fluorescence and mass spectrometry, represent a key advancement in analytical flexibility. Bao et al. developed a Zr-NMOF–aptamer probe, integrating zirconium MOFs with heterobifunctional aptamers [32]. This probe can simultaneously generate fluorescent and mass signals, significantly boosting sensitivity and data acquisition speed in IMC, while exhibiting specific molecular recognition. Its signal amplification exceeds that of commercial MCP probes by fourfold, making it suitable for high-plex co-staining. The Zr-NMOF–aptamer probe holds broad potential in spatial proteomics and is anticipated to become a next-generation molecular tool for mass cytometry. Huang et al. introduced PCN-224-OH, a luminescent material with dual fluorescent and mass detection capabilities, easily functionalized with antibodies or aptamers, as well as poly (ethylene glycol) (PEG) [33]. Probes like PCN-224-Ab-PEG and PCN-224-Apt-PEG enhance sensitivity and specificity, enabling rapid tissue scanning and reducing IMC blind scanning time by up to 90%. This work broadens the application of luminescent metal–organic frameworks and provides a novel toolbox for efficient single-cell multi-parameter analysis. Yu et al. developed lanthanide-doped fluorescent carbon nanodots (B/G/R-Cdots) as dual-modal tags for immunofluorescence microscopy (IFM) and IMC [34]. When conjugated with aptamers (e.g., B-Cdots(¹⁵⁹Tb)-A10-3.2), these probes enable identification of regions of interest (ROIs) via IFM fluorescence, reducing IMC blind scanning time by 90% in prostate cancer tissues. High-resolution protein mapping by IFM complements the lower resolution of IMC, highlighting the clinical potential for integrated imaging. This dual-modal probe, combining IMC and IFM holds great promise for clinical applications.

Modular surface chemistries are essential for minimizing nonspecific binding in complex biological environments. As mass cytometry tags, nanoparticles mitigate nonspecific interactions through multifaceted mechanisms: size exclusion limits off-target binding, tunable surface chemistries (e.g., PEGylation, zwitterionic coatings) reduce nonspecific adsorption, high metal payloads improve signal-to-noise ratios, and bioorthogonal ligands (e.g., aptamers, antibodies) enable target-specific recognition. Collectively, these properties enhance detection accuracy and reliability, establishing

nanoparticles as indispensable tools for precise biomolecular profiling in mass cytometry and imaging.

Liu and colleagues introduced a novel mass tag strategy using cost-effective polystyrene nanoparticles (PS-NPs) as metal carriers [35]. These submicron PS-NPs efficiently load metals and are detectable via mass cytometry, eliminating the need for ultra-small particles. Modifying cell staining buffers reduces nonspecific binding of unfunctionalized PS-NPs, offering an alternative to surface modification. Antibody-conjugated, metal-doped PS-NPs demonstrate high compatibility with commercial mass tags, achieving a fivefold increase in sensitivity. Additionally, four hafnium (Hf)-based detection channels (¹⁷⁷Hf–¹⁸⁰Hf) were incorporated. This work presents a scalable, cost-efficient mass tag platform and a buffer-modification method to reduce background signals, expanding multiplexing capabilities and enabling higher-throughput single-cell analyses in biomedicine.

Arnett and colleagues explored lanthanide nanoparticles (LnNPs) as high-sensitivity mass tags for mass cytometry immunoassays [36]. They synthesized 39 nm NaYF₄: Yb, Er nanoparticles coated with a biotinylated lipid formulation, characterized by dynamic light scattering (DLS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Using polystyrene microsphere models and suspension/IMC analysis, they demonstrated that lipid-coated LnNPs enable targeted binding with minimal nonspecific binding. The lipid composition significantly affects reporter performance, underscoring the importance of quantifying nanomaterial-lipid interactions for optimization. These findings position LnNPs as promising mass cytometry mass labels, highlighting the relevance of lipid formulation engineering and compositional analysis to improve specificity and sensitivity, with implications for single-cell proteomics and immunology.

Abtahi et al. synthesized NaYF₄: Yb³⁺/Er³⁺ nanoparticles for IMC, employing a two-step ligand exchange to coat nanoparticles with methoxy-PEG_{2K}-nelumonic acid (PEG-Ner) and/or poly (sulfobetaine methacrylate)-nelumonic acid (PSBMA-Ner) [37]. Coatings with PEG-Ner and PSBMA-Ner ensured colloidal stability in PBS. In tonsil tissue, PSBMA-Ner or their 1:1 mixture suppressed nonspecific

binding at 2×10^{10} nanoparticles/mL via steric and charge shielding, whereas PEG-Ner alone was ineffective. In breast cancer tissues, higher nanoparticle concentrations increased nonspecific binding, but combined coatings mitigated this effect. This study highlights heterobifunctional PEGs as a strategy to optimize nanoparticle surface chemistry for reduced nonspecific binding and improved spatial proteomics in clinical tissue analysis.

Nanoparticles capable of cellular internalization enable real-time monitoring of biological processes. Vancaeyzeele et al. synthesized ~ 100 nm monodisperse polystyrene nanoparticles (PS-NPs) chelated with approximately 1 000 lanthanide ions (single or mixed elements) [38]. These particles are internalized via nonspecific phagocytosis in three leukemia cell lines, allowing ICP-MS quantification of adhesion dynamics during PMA-induced THP-1 differentiation. The high ion payload enhances sensitivity, while label-free internalization simplifies protocols. This work demonstrates real-time tracking of complex cellular activities such as adhesion, positioning these nanoparticles as versatile tools for high-precision cell labeling and bioanalysis in cancer and immune research, supporting single-cell studies and mechanistic investigations. Schulz et al. utilized silver nanoparticles (AgNPs) for leukocyte antigen detection in mass cytometry [39]. Silver isotopes (107/109 Da) occupy exclusive mass channels, enabling specific detection of antigens such as CD8 and CD25. The 40 nm streptavidin-coated AgNPs effectively detected low-abundance CD25⁺ regulatory T cells with minimal nonspecific binding, compatible with existing protocols. Isotopically pure AgNPs

expand the panel parameters of mass cytometry, improving detection of rare cell populations. Y. Zhang et al. synthesized 5.7 nm amine-functionalized tantalum oxide nanoparticles via a one-pot two-step reverse microemulsion method Fig. 5 [40]. After azide modification and conjugation with DBCO (dibenzocyclooctyne)-functionalized antibody, these conjugates effectively detected CD20 on Ramos cells and supported high-dimensional PBMC analysis. This work advances the development of elemental and high-sensitivity mass tags for mass cytometry.

The sizes of MCPs and nanoparticle-based tags (10–1 000 nm) significantly influence detection sensitivity through steric hindrance and particle heterogeneity. Larger tags (e.g., >200 nm) are often obstructed by the glycocalyx or densely packed receptor arrays on the cell surface, whereas smaller tags (e.g., 10–20 nm) exhibit better access to cell-surface antigens due to reduced steric barriers [30]. For example, 13-nm monodisperse NaHoF₄ nanoparticles generate signals two orders of magnitude stronger than MCPs when binding to low-abundance antigens, demonstrating the advantages of smaller sizes [30]. Cellular uptake efficiency is also size-dependent: particles smaller than 100 nm are efficiently internalized via endocytosis, while those larger than 500 nm tend to be trapped in endosomes. This disparity arises from the higher surface-to-volume ratio of smaller particles, which enhances membrane interactions and facilitates cellular entry, whereas larger particles face hydrodynamic barriers that hinder endocytosis [41]. Beyond size, nanoparticles uniformity is critical; a non-uniform size distribution (coefficient of variation >15%) or

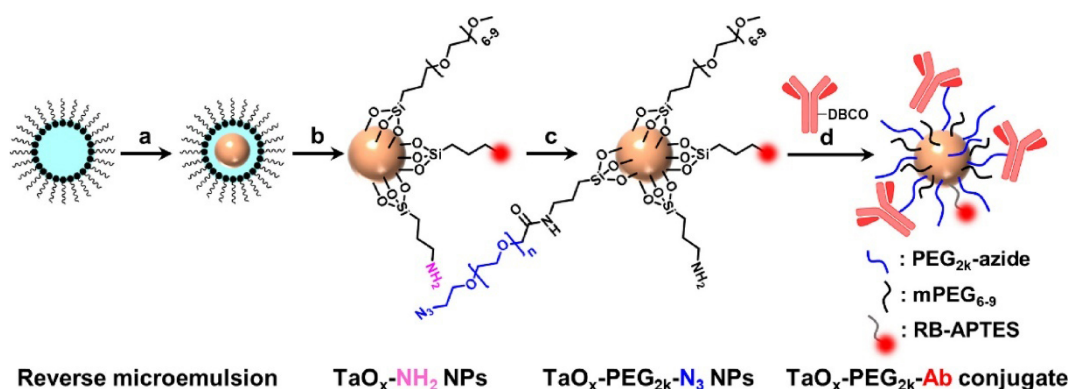


Fig. 5 Fabrication process of the TaO_x-PEG_{2k}-Ab conjugate. (a) Tantalum ethoxide was injected into the reverse microemulsion. (b) 3-aminopropyltriethoxysilane (APTES), rhodamine B-conjugated APTES (RB-APTES), and 2-methoxy(polyethyleneoxy)₆₋₉ propyltrimethoxysilane (mPEG₆₋₉) were sequentially added into the microemulsion. (c) TaO_x-NH₂ nanoparticles were reacted with NHS-PEG_{2k}-N₃ cross-linkers. (d) TaO_x-PEG_{2k}-N₃ nanoparticles were reacted with DBCO-functionalized antibodies. Reproduced with permission from Ref. [40] © 2020 American Chemical Society.

inconsistent metal loading can cause fluctuations in ionization efficiency and signal variability. Conversely monodisperse particles (CV <5%), achievable through methods like microfluidic synthesis, greatly improve signal stability by minimizing heterogeneity [42].

Nanoparticle-based tags have revolutionized mass cytometry by overcoming sensitivity hurdles through high metal payloads, multimodal imaging, and surface engineering. Future endeavors should focus on translating these systems into clinical diagnostics, optimizing suppression of nonspecific binding in heterogeneous tissues, and exploring their application in real-time monitoring of dynamic cellular processes (e.g., immune cell trafficking, tumor cell adhesion).

Microbeads

Flow cytometry and mass cytometry are crucial techniques for analyzing complex cellular systems at the single-cell resolution. Traditional flow cytometry employs microbeads for instrument calibration and quantitative analysis, but these fluorescence-based beads are incompatible with mass cytometry's mass-based detection method [43]. Existing metal-containing microbeads often lack suitability for immunoassays.

To address this, Budzinski et al. developed osmium tetroxide (OsO_4)-labeled microbeads, enabling distinct mass signal detection [44]. This innovation enhances spillover compensation and quantification in mass cytometry, allowing accurate measurement of antibody binding and receptor expression in human PBMCs.

Polystyrene microbeads are favored in mass cytometry due to their narrow size distribution (1–5 μm) and capacity to incorporate 10^4 – 10^8 metal ions per bead, ensuring detectable signals. Abdelrahman, et al. created lanthanide-loaded polystyrene beads for calibration and internal standards, demonstrating stable metal incorporation and reliable cellular measurements [45]. Finck et al. embedded lanthanide metals into polystyrene microbeads to serve as internal standards and developed algorithm to correct for instrument-induced signal fluctuations, reducing signal variability from 4.9-fold to 1.3-fold in longitudinal studies [46]. Moreover, Liu et al. introduced polymerizable metal complex to control metal loading, allowing consistent incorporation of various ions for mass cytometry (Fig. 6) [47]. They also developed seven-element-encoded microbeads, enhancing multiplexing capacity and calibration accuracy.

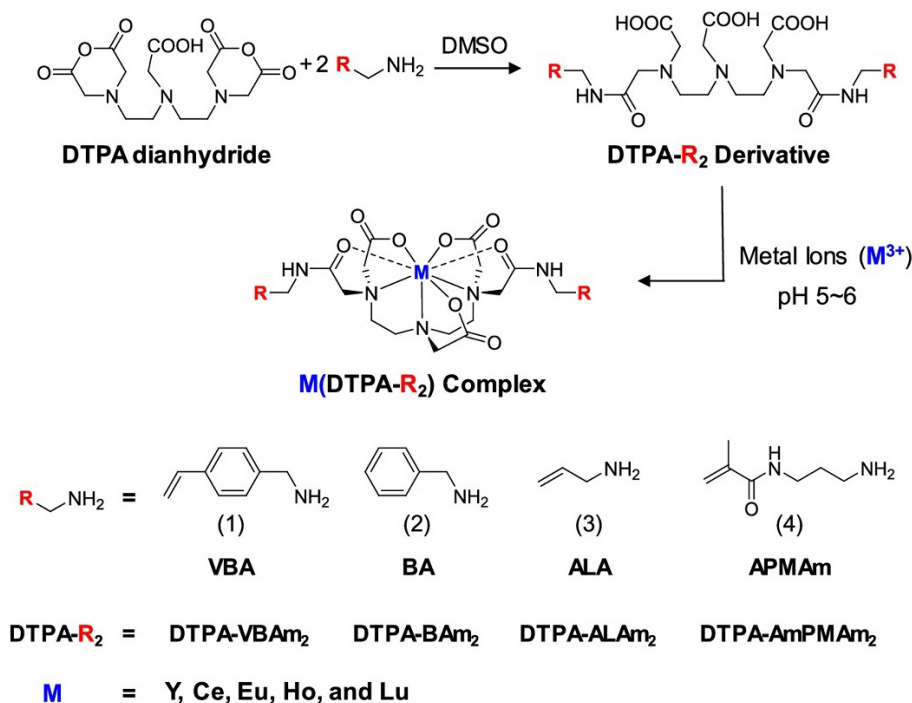


Fig. 6 The synthesis of DTPA derivative-metal complexes for two-stage dispersion polymerization. The metal chelator DTPA was functionalized via reaction of DTPA anhydride with amine-containing functional molecules in DMSO. Various metal ions were loaded onto DTPA derivatives at pH 5–6. Reproduced with permission from Ref. [47] © 2021 American Chemical Society.

Additionally, Bringeland et al. utilized standardized quantum single-stranded beads (QSCS) to correct detection sensitivity variations, improving measurement accuracy of receptor occupancy in mass cytometry [48]. These advancements collectively improve calibration, normalization, and signal reliability in cellular analyses.

The progress in microbead technology for mass cytometry significantly enhances detection accuracy and data reliability. Innovations like osmium-labeled beads, controlled metal-loading polystyrene beads, and multi-element encoding extend the capabilities of mass cytometry beyond traditional fluorescence methods. These developments address challenges such as signal variability and sensitivity, facilitating high-dimensional analysis of cellular interactions and functions. Ultimately, these efforts advance our understanding of complex biological systems in biomedical research.

Mass tag cellular barcoding

Mass tag cellular barcoding (MCB) is an innovative technique that employs uniquely isotopically pure mass tag reagents to encode diverse sample types for analysis. In this approach, barcoded samples are pooled, stained with antibody panels, and analyzed via CyTOF. MCB plays a crucial role in mass cytometry by reducing procedural variations, increasing throughput, and enabling the simultaneous processing of multiple samples [49].

Palladium isotopes are commonly used MCB reagents; however, they generally exhibit lower signal intensities compared to cadmium isotopes, limiting their effectiveness in high-throughput settings. To address this, Muftuoglu et al. developed a novel barcoding strategy combining seven cadmium (Cd) and three palladium (Pd) isotope tags, resulting in ten distinct labels [50]. These tags provide high signal intensity without interfering with lanthanide detection, thereby improving multiplexing and throughput. Notably, this study utilized MCP9 polymer to chelate the isotopes, a common approach in traditional metal-chelating systems.

Nevertheless, the stability of metal ions in complex biological environments remains a significant challenge. As demonstrated in the literature, metal ions in conventional MCPs are susceptible to dissociation due to competitive binding with proteins or pH fluctuations in biological media, which

diminishes detection sensitivity [30]. In contrast, nanoparticles such as NaHoF₄ leverage an inorganic lattice to stabilize metal ions, achieving signal intensities two orders of magnitude higher than MCPs while maintaining superior stability [30]. This mechanistic advantage addresses a core limitation of early MCP-based systems, paving the way for nanomaterial-driven innovations in MCB.

To mitigate nonspecific binding, another key challenge in cellular barcoding, strategies such as nanoparticle surface functionalization, control of monodispersity, and size optimization have been developed. Surface modifications like PEGylation or phospholipid coatings reduce nonspecific interactions through steric hindrance and charge neutralization. For example, NaYF₄ nanoparticles encapsulated within a DEC221 lipid mixture (DOPC:ESM:cholesterol = 2:2:1) and conjugated with mPEG2K-DSPE exhibit nonspecific binding levels below 0.1% of the applied dose [42]. Furthermore, monodisperse nanoparticles (with coefficient of variation, CV < 5%) ensure consistent ionization efficiency, while particle sizes of 10–20 nm facilitate access to cell-surface antigens by minimizing steric barriers posed by the glycocalyx.

Building on these advances, Zunder et al. introduced Pd-based labeling reagents to expand measurement channels and reduce interference with lanthanide detection [51]. They implemented an error-detection combinatorial barcoding scheme to identify and remove cell doublets and developed a debarcoding algorithm that enhances accuracy using single-cell data. Their protocol modifications—including pre-permeabilization barcode staining—improved assessment of methanol-sensitive surface markers. Additionally, Delgado-Gonzalez et al. developed a fluorescent palladium-based hybrid label nanotracker for long-term live-cell barcoding, compatible with both flow and mass cytometry [52]. This method employs polystyrene nanoparticles labeled with palladium isotopes and fluorescent dyes, demonstrating suitability for long-term drug response studies.

Recent innovations in cell barcoding have further advanced mass cytometry. McCarthy et al. introduced a rapid cell barcoding approach using monoisotopic cisplatin, enabling labeling without cell permeabilization within 10 minutes [53]. This technique improves throughput and consistency while

preserving antibody measurement channels. Hartmann et al. developed a universal, antibody-based live-cell barcoding platform targeting the MHC class I and the sodium-potassium ATPase, allowing multiplex labeling of various human cell types [54]. Mei used multiple anti-CD45 antibody conjugates to barcode up to 20 PBMC samples, reducing technical noise and enhancing accuracy in cytometry-based immunophenotyping [55]. Bodenmiller et al. applied MCB in a 96-well format to study signaling dynamics in PBMCs, generating extensive phosphorylation data and detecting off-target effects in drug screening [56]. Catena et al. presented a multiplex labeling method using osmium and ruthenium, expanding the number of analyzable mass channels [57]. Willis designed a tellurium-based reagent, TeMal, capable of labeling both live and fixed cells for large-scale applications [58]. Wu et al. proposed a ratiometric barcoding strategy utilizing lanthanide-coordinated polymer dots (Ln-Pdots) [59], and Liu et al. explored the use of MOFs, achieving 84 barcode combinations via nonspecific binding of UIO-66 to cells [60]. The UIO-66 barcodes showed high compatibility with MCP mass tags and significantly improved sensitivity.

Collectively, these advancements substantially increase the throughput and accuracy of single-cell analysis, facilitating applications in drug discovery and personalized medicine.

Technical optimization strategies

Mass cytometry, particularly utilizing CyTOF technology, has become a powerful approach for high-dimensional analysis of biological samples, especially in immunology and clinical hematology. To fully realize its potential, various optimization strategies can be applied at different stages of the workflow, including experimental design, sample processing, antibody selection and labeling, instrument configuration, and data analysis. These optimizations aim to improve the accuracy, sensitivity, and throughput of mass cytometry, ultimately yielding more reliable and efficient results for addressing key biological questions.

A notable advancement in this field is the development of semi-automated data processing workflows, such as the one introduced by Rybakowska et al. [61]. This workflow leverages the R programming language and integrates multiple tools to reduce experimental bias and batch effects,

thereby enhancing data quality. It is particularly suitable for large-scale, multi-center studies, as it facilitates the processing and analysis of extensive immune monitoring datasets. Another significant contribution is from Liu et al., who designed a streamlined mass cytometry workflow tailored for clinical applications [62]. This workflow incorporates cryopreservation techniques and optimized barcoding strategies to minimize batch effects, improving the reliability of data obtained from human circulating immune cells. Their work highlights the critical role of sample handling and processing in reducing variability and enhancing the robustness of mass cytometry applications.

Despite its advantages, mass cytometry encounters challenges such as nonlinear spillover at high signal intensities, which can impair data interpretation. To address this issue, Sekhri et al. proposed a method that combines unlabeled competitor antibodies with metal-labeled antibodies to reduce spillover effects [63]. This approach improves staining accuracy and offers a more stable alternative to traditional antibody titration methods, thereby enhancing the overall reliability of mass cytometry data.

Panel design is a crucial aspect that must be carefully considered to ensure data consistency and reproducibility. Takahashi et al. highlighted the impact of signal interference on staining profiles and demonstrated that using mass-minus-one (MMO) controls can improve antibody performance consistency [64]. Their findings underscore the importance of control methods that effectively distinguish true signals from background noise, thereby improving data quality. Additionally, Tricot et al. revealed significant variability in lanthanide detection sensitivity across different mass ranges and instruments [65]. This variability underscores the need for careful selection of lanthanide combinations when designing staining panels, especially in multicenter studies. They advocate for the development of new reagents and normalization algorithms to address these differences, emphasizing the importance of standardization in mass cytometry.

Finally, the development of computational methods, such as DGCyTOF introduced by Cheng et al., offers a promising approach for improving cell population identification in single-cell mass cytometry data [66]. By combining deep learning with hierarchical clustering, DGCyTOF enhances

both the accuracy and speed of cell type classification while providing robust visualization tools. This innovative approach not only improves mass cytometry data analysis but also has the potential to be extended to other omics modalities, such as single-cell RNA sequencing.

In summary, the discussed optimization strategies underscore ongoing advancements in mass cytometry techniques and methodologies, which collectively aim to improve data quality, reliability, and applicability in biomedical research. Continued innovation in workflows, panel design, and computational analysis is expected to further solidify mass cytometry's role as a vital tool for understanding complex biological systems.

Advances in CyTOF and IMC for Enhanced Biomolecular Profiling

Recent innovations in mass cytometry and mass spectrometry have significantly advanced single-cell biomolecular analysis. CyTOF employs metal isotope-labeled antibodies to measure numerous proteins, including surface markers and signaling molecules. For example, Fagerholt et al. demonstrated CyTOF's ability to detect p53 protein and modifications in both normal and malignant cells [67].

Mass spectrometry innovations, such as Hall's

mass defect tags, facilitate rapid protein identification by enabling precise differentiation between labeled and unlabeled biomolecules, streamlining proteomics workflows [68]. Long's AdductHunter provides automated detection of protein-metal adducts, deepening understanding of therapeutics interactions [69]. R. Hong and colleagues addressed the limitations of traditional tissue mass spectrometry imaging (MSI) by introducing enzyme-amplified mass tags, enabling detection of protein biomarkers in formalin-fixed, paraffin-embedded (FFPE) samples without requiring protein digestion (Fig. 7) [70]. This innovation markedly enhances sensitivity and spatial resolution, thereby advancing applications in personalized medicine.

Warren et al. developed a novel workflow employing IMC to analyze mitochondrial protein distribution in skeletal muscle biopsies from patients with mitochondrial diseases [71]. Their approach allows for the identification of oxidative phosphorylation defects at the single-cell level, providing critical insights into disease mechanisms and potential therapeutic targets. Further advancements include scalable methods for conjugating immunoglobulins with stable mass isotopic reporters introduced by F.J. Hartmann and colleagues, which enhance the efficiency of single-cell mass cytometry [72]. Duckworth's PLAYR technology integrates RNA detection with protein

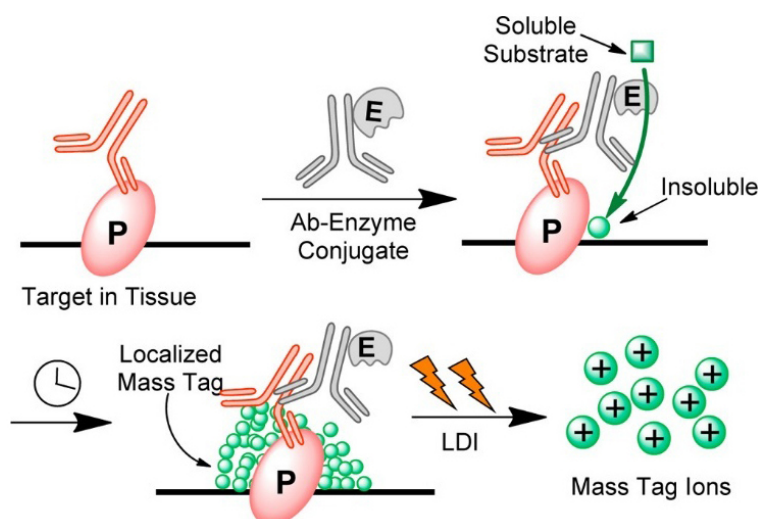


Fig. 7 The enzymatic amplification process of mass tags for targeted tissue imaging. A precursor to the mass tag is deposited near the target of interest via immuno-binding. The precursor then undergoes an enzyme-catalyzed transformation, converting a soluble substrate into an insoluble precipitate. This process leads to the accumulation of a substantial quantity of mass tags at the target site, thereby amplifying the signal. The localized mass tags are subsequently detected using laser desorption ionization mass spectrometry (LDI-MS), enabling the identification of the target within formalin-fixed paraffin-embedded (FFPE) tissue samples. Reproduced with permission from Ref. [70] © 2014 American Chemical Society.

analysis, enabling simultaneous measurement of RNA and protein expression—overcoming limitations of traditional methods [73]. Additionally, Mavropoulos and colleagues developed metal in situ hybridization, combining mass cytometry with RNA detection to analyze both proteins and mRNA within the same cells, thereby improving the study of gene expression and protein localization [74]. The amplification by cyclic extension (ACE) technique developed by Lun significantly increases the sensitivity of mass cytometry for detecting low-abundance proteins (Fig. 8), with broad applications in cellular signaling pathway analysis and tissue imaging [75]. Behbehani and colleagues utilized single-cell mass cytometry to quantify cell cycle stages, effectively distinguishing among different phases in both normal and cancerous cells [76]. This method allows comprehensive immunophenotyping and cell cycle analysis within a single experiment, providing valuable insights into the hematopoietic hierarchy in healthy human bone marrow.

The integration of advanced imaging technologies has substantially enhanced our capacity to analyze complex biological systems at the single-cell level. A notable example is the innovative strategy developed by Fusco and colleagues, named “Label-free sINgle-cell tracKING of 2D matERials by mass cytometry and multiplexed ion beam imaging-time of flight (MIBI-TOF) design (LINKED)” [77]. This approach combines CyTOF and MIBI-TOF to enable label-free detection of 2D materials, specifically transition

metal carbides, nitrides, and carbonitrides (MXenes), within human immune cells. Their findings demonstrated successful quantification of various MXenes across 15 immune cell subsets, along with assessments of biocompatibility and effects on cellular interactions. *In vivo* biodistribution studies confirmed MXenes accumulation in key organs, underscoring the technology’s potential to reveal new insights into cellular dynamics and material interactions in biomedical applications.

Schlecht and colleagues applied IMC to perform high-dimensional analyses of ocular tissues [78]. By profiling 18 biomarkers in formalin-fixed, paraffin-embedded conjunctival tissues, they identified 24 distinct cell clusters through bioinformatics analysis. This highly multiplexed imaging at subcellular resolution provides valuable insights into the pathogenesis of ocular diseases, highlighting IMC’s relevance in basic research, drug development, and clinical diagnostics.

Furthermore, the application of Hyperion IMC to Becker muscular dystrophy (BMD) by Piñol-Jurado and colleagues has provided new insights into the disease progression [79]. By analyzing muscle biopsy samples from patients at various stages of BMD, they found significant correlations between collagen-I levels and other tissue components, revealing structural differences between fibrotic and non-fibrotic regions. This study emphasizes IMC’s utility in elucidating complex tissue architecture and

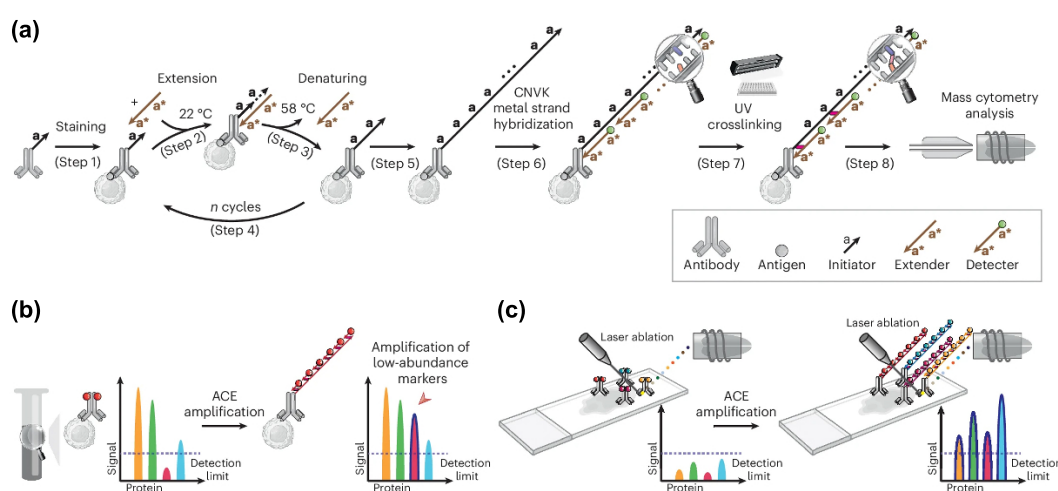


Fig. 8 the schematic of ACE and its applications. **(a)** The ACE approach for signal amplification in mass cytometry analysis. **(b)** ACE amplifies signal in suspension mass cytometry, enabling the detection and quantification of low-abundance markers that fall below detection limits of conventional mass cytometer techniques. **(c)** When combined with IMC, ACE facilitates high-sensitivity, multiparametric spatial profiling of healthy and diseased tissue samples. Reproduced with permission from Ref. [75] © 2024 The Authors.

spatiotemporal relationships during disease progression, advancing the discovery of novel pathogenic pathways in human tissues.

These studies exemplify the transformative impact of advanced imaging techniques, such as mass cytometry and IMC, in biomedical research. They enable detailed analysis of cellular and tissue dynamics, advancing our understanding of disease mechanisms and informing diagnostics and therapies. The ability to visualize and quantify biomolecular interactions at unprecedented resolutions opens new avenues for research and clinical applications, underscoring the importance of integrating innovative technologies in biomedical engineering. Such integration supports comprehensive analyses that can significantly influence personalized medicine and therapeutic development.

Applications of Mass Cytometry in Biomedical Research

Mass cytometry has broad applications across diverse biomedical fields, including immunology, oncology, neurology, hematology, stem cell research, infectious diseases, and drug development. This technology generates high-dimensional data, enabling detailed characterization of multiple cell subpopulations within a single sample. It facilitates the study of heterogeneity within known cell populations, identification of changes associated with clinical states, assessment of similarities between target and

reference cell populations, detection of physiological state transitions, and delineation of intermediate or branching developmental states.

Immunology

Immunology investigates the structure and function of the immune system, where immune cells are essential for health maintenance and pathogen defense. Mass cytometry has numerous applications in immunology, primarily including immune cell phenotyping and classification, immune responses analysis, diagnosis of immune disorders, and tumor immunology research.

Immune cell phenotyping

Immune cells such as T cells, B cells, natural killer (NK) cells, and dendritic cells (DCs) exhibit considerable heterogeneity. Traditional classification methods primarily rely on surface marker detection, which limits the number of parameters measured simultaneously, restricting comprehensive analysis of immune cell subsets. CyTOF overcomes this limitation by enabling the concurrent detection of multiple surface markers, thereby improving the precision and depth of immune profiling.

Recent advancements in mass cytometry have yielded innovative methodologies for immune cell analysis. For example, as depicted in Fig. 9, Stensland et al. developed a multiplexed enrichment technique employing cleavable magnetic nanoparticles to isolate antigen-reactive B cells—such as those responsive to insulin and tetanus toxoid [80]. This method allows efficient monitoring

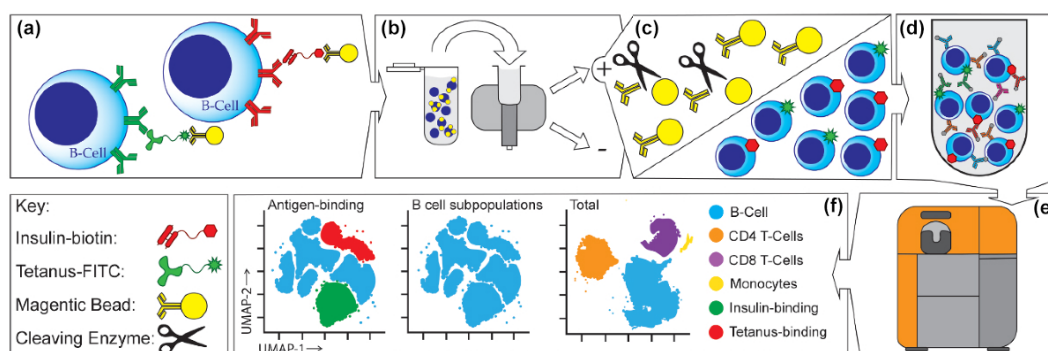


Fig. 9 Schematic for magnetic enrichment of antigen-reactive B cells. (a) Human PBMCs are first incubated with precisely titrated amounts of biotin and FITC-labeled antigens. Subsequently, the cells are treated with a combination of cleavable anti-biotin and anti-FITC magnetic beads. (b) After washing, the cells are loaded into a magnetic column, separating into depleted and enriched fractions. (c) The magnetic microbeads are cleaved off and washed away, leaving the enriched cells population. (d) The cells are then incubated with a cocktail of metal-labeled antibodies. (e) The enriched cells are analyzed using a mass cytometer. (f) Data are processed through manual gating, dimensionality reduction, and clustering algorithms. Reproduced with permission from Ref. [80] © 2021 The Authors.

of B cell phenotype and function during disease progression or vaccination, while reducing sample volume and processing time. Stevens et al. introduced a method for quantifying target expression on circulating immune cells in whole blood using CyTOF [81]. By determining the maximum antibody binding capacity (Bmax) and converting it to the number of antibodies bound per cell (ABC), they successfully quantified CD4 and CD8 expression on T cells, obtaining results consistent with traditional flow cytometry. This approach also allowed multiplexed measurements of various immune markers across multiple cell subsets. In the context of diabetic nephropathy (DN), Jin et al. used CyTOF to analyze immune heterogeneity in patients with Type II diabetes [82]. Their findings revealed significant alterations, including reduced B cell counts and increased monocyte levels, along with potential biomarkers associated with DN progression. These insights shed light on immune-driven mechanisms underlying DN and suggest new immunotherapy targets. Koppejan et al. explored mass cytometry's utility for immune monitoring in patients with axial spondyloarthritis (axSpA) [83]. Longitudinal analysis of PBMC samples revealed distinct immune profiles between patients and controls, with immune cell cluster changes correlating with disease activity—highlighting mass cytometry's potential in clinical and longitudinal studies. Additionally, van der Pan et al. compared spectral flow cytometry (SFC) and mass cytometry in analyzing innate myeloid cells [84], finding both methods comparable in identifying and characterizing IMCs, though with minor differences in variability and reproducibility. This comparison enhances understanding of each method's strengths and limitations in complex immune populations. Shi et al. developed a spatial analysis workflow to investigate IMC data from cardiac tissue post-myocardial infarction [85], revealing spatial gradients of immune reactivity and neutrophil density, thus providing insights into neutrophil-mediated injury mechanisms. This innovative spatial approach underscores mass cytometry's potential for elucidating immune responses within tissue microenvironments.

Overall, mass cytometry significantly advances immune cell phenotyping, offering comprehensive insights into immune diversity and function. Its versatile applications across numerous diseases contribute to understanding immune mechanisms and

identifying therapeutic targets. The integration of novel methodologies and spatial analyses further enhances our understanding of immune dynamics in health and disease.

Study of immune responses

Immune responses are complex reactions initiated by the body to combat pathogens or other stimuli. Mass cytometry has emerged as a powerful tool for examining the activation, proliferation, and differentiation of immune cells during these processes.

Stroke is a major global health issue with limited treatment options. Angong Niu Huang Wan (AGNHW), a traditional Chinese medicine, exhibits neuroprotective effects, although its mechanisms remain unclear. Yao and colleagues utilizing CyTOF demonstrated that AGNHW reduces monocyte-derived macrophages infiltration into the brain and decreases inflammatory markers, thereby mitigating post-stroke inflammation [86]. Additionally, AGNHW modulates various immune cell populations in peripheral blood and inhibits key inflammatory markers, indicating its potential therapeutic role in ischemic brain injury.

Rejection remains a significant obstacle in solid organ transplantation, often resulting in graft loss. Single-cell immune profiling techniques, such as IMC, provide insights into the immunological mechanisms underlying rejection. Ung et al. developed an IMC panel to analyze chronic rejection in liver transplant samples, identifying unique immune cell subpopulations and illustrating the utility of single-cell data in building predictive models [87]. This underscores the potential of IMC in discovering novel biomarkers and therapeutic targets.

Microgravity during spaceflight has been associated with immune suppression and increased viral shedding, however, the underlying mechanisms are not fully understood. Spatz and colleagues used single-cell mass cytometry to analyze immune responses under simulated microgravity conditions [88]. Their findings revealed suppressed activity of cytotoxic immune cells and enhanced regulatory T cell functions, indicating dual effects on the immune system. This study provides a framework for understanding immune adaptation during long-term space missions.

Recent investigations have explored interactions

between two-dimensional transition metal dichalcogenides (TMDs), such as molybdenum disulfide (MoS_2) and tungsten disulfide (WS_2), and immune cells. Advanced analytical methods tracked their biodistribution and effects on immune cell function [89]. Results suggest TMDs exert less impact on immune cell viability than graphene and are trackable *in vivo*. Notably, WS_2 exhibited superior brightness and signal intensity, highlighting its potential as an immunocompatible nanoplatform for biomedical applications.

Advances in single-cell techniques like mass cytometry and IMC have markedly enhanced our understanding of immune responses across biomedical contexts. These methods enable detailed profiling of immune cell populations and their functional states, paving the way for novel therapies and improved patient outcomes. Moreover, exploring immunomodulatory agents and integrating traditional medicine with nanotechnology hold promise for future biomedical innovations.

Diagnosis of immune diseases

Immune diseases result from dysfunctional immune responses, including autoimmune disorders, allergies, and immunodeficiencies. Mass cytometry is a powerful diagnostic tool that detects abnormal immune cell populations by analyzing marker expression profiles. This technique can improve the diagnosis and understanding of immune-related conditions.

Myasthenia Gravis (MG) is characterized by autoantibodies targeting the neuromuscular junction, especially the acetylcholine receptor (AChR). Verdier et al. utilized single-cell mass cytometry to analyze PBMCs from MG patients [90]. The observed reductions in monocyte subsets and increases in type 2 innate lymphoid cells (ILC2s) and CD27- $\gamma\delta$ T cells, indicating complex immune dysregulation involving both innate and adaptive responses. Childhood asthma, prevalent in industrialized areas, is linked to environmental factors such as farm dust, which may confer protective effect. Beerweiler and colleagues employed CyTOF to investigate immune regulation following farm dust exposure [91]. Their results showed downregulated of innate immune cells and upregulating of adaptive immune cells, offering insights into immune mechanisms related to asthma prevention.

Cellular senescence contributes to aging and age-related diseases. Due to heterogeneous marker expression, identifying senescent cells is challenging. Li et al. developed a CyTOF-based framework for detecting senescent cells in skeletal muscle, revealing the important role of autophagy-related proteins [92]. Their findings highlight the cellular heterogeneity of senescence and suggest potential therapeutic targets for age-associated conditions. Celiac disease (CeD), an immune-mediated disorder triggered by gluten, has been traditionally studied with limited resolution. stevam et al. implemented a high-parameter CyTOF workflow, enabling comprehensive analysis of immune changes in PBMCs from CeD patients [93]. This approach enhances understanding of immune responses in CeD and may inform treatment strategies. Inflammatory Bowel Diseases (IBD) involves abnormal immune activity in the gastrointestinal tract. van Unen et al. used high-dimensional mass cytometry to identify 142 immune cell subsets in healthy and diseased tissues [94]. Their analysis facilitated differentiation between patients and controls and assisted in detecting mucosal lymphoid malignancies, contributing to improved diagnostic and targeted therapies.

The progressive development of mass cytometry and high-dimensional analysis techniques is revolutionizing the diagnosis and comprehension of immune diseases. These methods provide detailed cellular insights, supporting accurate diagnosis, personalized treatment, and better patient outcomes.

Tumor immunology

CyTOF is instrumental in analyzing immune cell composition and function within the TME, aiding in the discovery of new targets and strategies for cancer immunotherapy.

High-grade B-cell lymphomas, such as Double Hit Lymphoma (DHL) and Triple Hit Lymphoma (THL), are aggressive and respond poorly to standard treatments. Recent studies using CyTOF have characterized the immune profiles of these lymphomas. For example, Lei et al. analyzed peripheral blood immune cells in DHL patients, revealing increased double-positive T cells and decreased double-negative T cells compared to diffuse large B-cell lymphoma (DLBCL) and healthy controls [95]. Elevated CD38 expression in naïve B cells was also identified as a potential biomarker or

therapeutic target. In acute myeloid leukemia (AML), measurable residual disease (MRD) after remission indicates higher relapse risk. Sefland et al. used CyTOF to profile immune cells in AML patients with MRD. They observed reduced CD57⁺ natural killer (NK) cells and increased activity/maturation markers in T and NK cells, suggesting that immune profiling could serve as a biomarker platform for monitoring remission [96]. Furthermore, A. J. Lamble et al. combined functional and phenotypic CyTOF analyses to characterize the AML immune microenvironment, aiming to understand T cell dysfunction and immune evasion mechanisms. This integrated approach provides a foundation for developing immune-based therapies [97].

Oncology

Cancer remains a significant global health threat, primarily due to tumor heterogeneity and the complexity of the TME. The TME is a dynamic and intricate system comprising cancer cells, immune cells, stromal cells, extracellular matrix components, and cytokines, all contributing to tumor progression and treatment resistance. Recent advances have heightened interest in the TME, especially regarding its role in immune evasion and therapeutic resistance, notably in hematological malignancies. However, the TME remains incompletely characterized, and inter-patient variability in tumor phenotypes challenges the development of effective treatments. For example, Gonder et al. utilized CyTOF technology to analyze the TME in chronic lymphocytic leukemia (CLL), identifying key immune checkpoints such as LAG3 and PD-1, thereby supporting the potential of TME-targeted immunotherapies [98].

To better understanding the TME, innovative imaging techniques are essential. IMC enables simultaneous detection of multiple antigens and transcripts, although traditional limited to 2D imaging. As illustrated in Fig. 10, Kuett et al. developed a three-dimensional (3D) IMC approach that integrates data from serial sections to create comprehensive tissue models [99]. This method has been successfully applied to human breast cancer samples, providing insights into cellular heterogeneity and tissue architecture that are not observable with 2D imaging.

Pancreatic ductal adenocarcinoma (PDAC) exemplifies a cancer type wherein the TME critically

influences disease progression and treatment outcomes. Research by Erreni et al. employed preclinical models to analyze the PDAC TME, developing a panel of 28 markers for single-cell IMC analysis [100]. Their findings clarified the spatial distribution and interactions among TME cells, offering valuable tools for understanding PDAC complexity and resistance mechanisms. Additionally, mass cytometry provides a robust platform for investigating tumor heterogeneity by allowing the detection of multiple markers simultaneously. This is particularly relevant for embryonic antigens (EAs), which are generally suppressed in adult somatic cells but can be re-expressed in cancers. Aanei et al. explored EAs expression in acute myeloid leukemia (AML), revealing that EAs are often downregulated in immature myeloid cells [101]. Their results suggest that analyzing EAs and related signaling pathways could identify unique phenotypes and assist in patient monitoring.

In summary, investigating the TME alongside advanced imaging techniques like 3D IMC is vital for elucidating tumor biology and improving therapeutic strategies. Incorporating mass cytometry to study tumor heterogeneity and EA expression across cancers, such as AML and PDAC underscores the potential for targeted therapies tailored to individual tumors characteristics. Continued research in these areas is critical for advancing cancer treatment and enhancing patient outcomes.

Neurology

The development of neuronal and glial populations in the dorsal root ganglia (DRG) is essential for detecting touch, proprioception, temperature, and noxious stimuli. Although previous studies have documented sex differences in somatosensory perception, there has been limited research on overall sex differences in DRG cell type abundance and developmental origins. To address this gap, Vradenburgh et al. employed CyTOF to investigate sexual dimorphism in neonatal mouse DRG [102]. Their findings revealed that at postnatal day 0 (P₀), females exhibited significantly higher numbers of total neurons, including TrkB⁺ and TrkC⁺ neurons involved in mechanosensation and proprioception, compared to males. Conversely males showed a greater abundance of TrkA⁺ neurons associated with thermosensation and nociception. Pseudotime analysis further indicated that neurons in males were

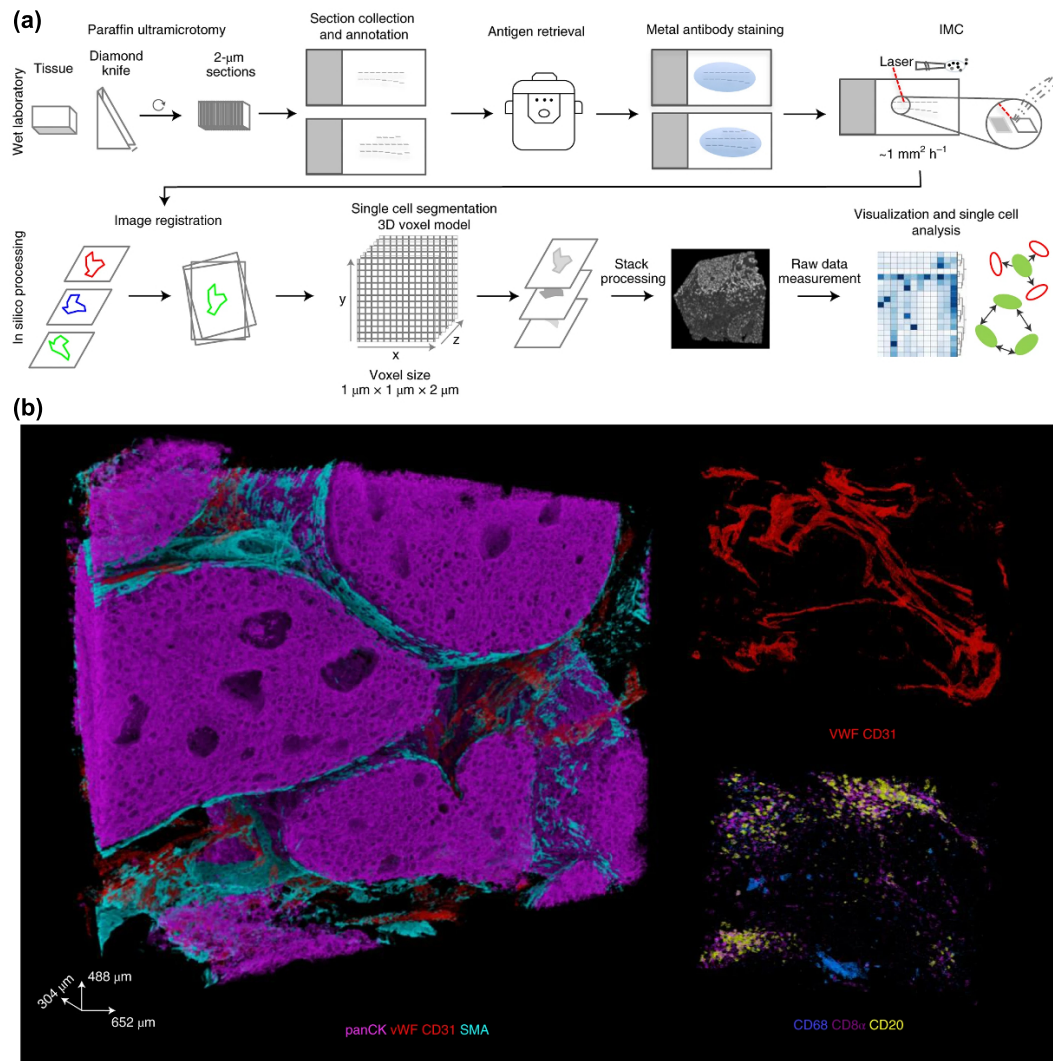


Fig. 10 Experimental and computational workflow for 3D IHC. **(a)** Small rods or blocks of formalin-fixed, FFPE tissue are sectioned into 2-μm slices using an ultramicrotome equipped with a modified diamond knife. Serial sections are collected on standard microscopy slides, typically 20 to 40 sections per slide. After rehydration, tissues undergo antigen retrieval, followed by staining with metal-labeled antibodies. The sections are then analyzed via IMC. The resulting data are processed computationally to organize sections according to their annotations. Images are aligned, and cells are segmented using a 3D watershed algorithm. The complete 3D model can then be examined at both voxel and cell levels. **(b)** Example of raw voxel data rendering for selected markers, derived from one of two breast carcinoma 3D IHC models. Data visualization was performed using AGAVE 1.0.0.1 software. Reproduced from Ref. [99] © 2021 The Authors.

more mature and differentiated than those in females. These results suggest sex-specific differences in DRG development and provide a foundation for further studies on their persistence into adulthood and effects on tactile perception.

Microglia, the resident immune cells in the central nervous system (CNS), are vital for brain development and homeostasis, as well as for involvement in neurological diseases. However, research on human microglia remains limited. Fernández-Zapata et al. provided a comprehensive review on the applications of CyTOF and IMC in studying human microglia (huMG) [103]. CyTOF

enables high-dimensional protein expression analysis of huMG, which, combined with single-cell RNA sequencing, offers detailed insights into cellular signaling pathways. IMC provides spatial information from tissue sections through mass spectrometry, enabling the analysis of cellular interactions. These technologies are powerful tools for investigating microglial heterogeneity and functions in both healthy and diseased human brain tissue.

Infectious diseases

Sepsis, a life-threatening organ dysfunction caused by dysregulated host responses to systemic microbial infection, presents challenges for traditional flow

cytometry. Advances in mass cytometry, especially CyTOF, enable comprehensive single-cell immune profiling in sepsis.

In a study by Park et al., PBMCs from 20 sepsis patients and 10 healthy donors were analyzed using CyTOF with 23 markers [104]. The study employed manual gating, automated clustering (PhenoGraph), and Uniform Manifold Approximation and Projection (UMAP) for enhanced cell identification and visualization (Fig. 11). Results showed significant alterations in immune cell populations in sepsis, including increased monocytes, B cells, and dendritic cells, and decreased natural killer cells and CD4/CD8 T cells. Distinct subpopulations were identified, with markers such as CCR6 exhibiting increased expression in specific monocyte subsets. These findings suggest potential novel diagnostic markers for sepsis and lay the groundwork for improved early detection and intervention strategies.

Additionally, Calderon-Gonzalez, et al. developed Bac-CyTOF, a method combining mass cytometry with bacterial tracking to study host-pathogen interactions *in vivo*, focusing on *Klebsiella pneumoniae* pneumonia in mice [105]. This approach revealed a pulmonary immune landscape characterized by increased neutrophils and inflammatory monocytes expressing PD-L1, alongside decreased alveolar macrophages. The bacterium's Type VI secretion system (T6SS) significantly influenced interactions with monocytes and macrophages, affecting inflammatory responses. The study also demonstrated that deficiency in the DNA sensor STING enhances bacterial clearance,

suggesting that *K. pneumoniae* may exploit STING signaling for survival.

These studies underscore the importance of advanced cell counting techniques in understanding the immune responses of infectious diseases, particularly sepsis, and provide new avenues for diagnosis and treatment.

Drug development

Osteoarthritis (OA) is a complex disease currently lacking effective disease-modifying treatments, partly due to patient heterogeneity causing many candidate drugs to fail in clinical trials. To address this challenge, Sahu and colleagues developed a CyTOF platform to evaluate the effects of preclinical drugs on human OA chondrocytes [106]. As shown in Fig. 12, chondrocytes were obtained from patients undergoing total knee replacement surgery and treated with either a control (DMSO), the NF- κ B pathway inhibitor BMS-345541, or the chondrogenic small molecule kartogenin. CyTOF analysis demonstrated that BMS-345541 significantly reduced the expression of key inflammatory and senescence-associated markers across multiple chondrocyte clusters, indicating a consistent drug response among patients. Conversely, kartogenin had a more limited effect, influencing fewer chondroprogenitor populations.

Yang and colleagues illustrated the utility of mass cytometry as a high-throughput, label-free method for analyzing the biodistribution of inorganic nanoparticles at the single-cell level [107]. Their results showed that mass cytometry could detect as

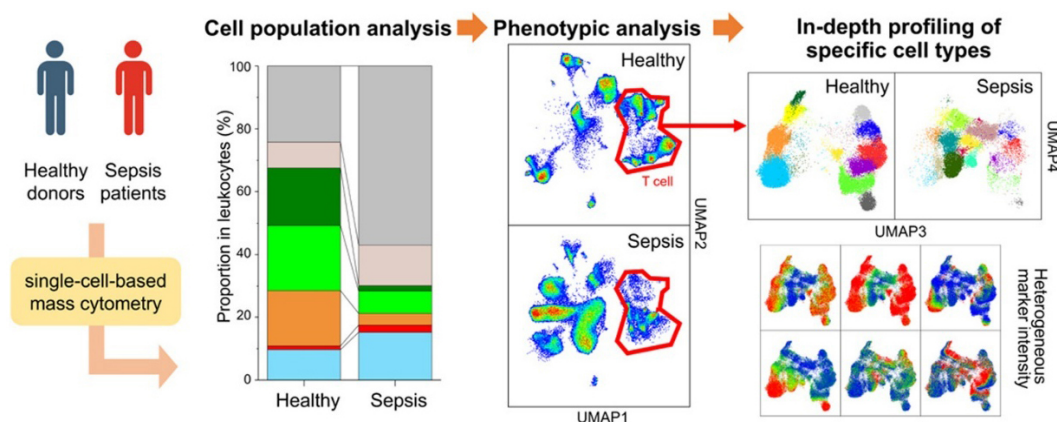


Fig. 11 A single-cell mass cytometry-based phenotyping analysis of immune cells in sepsis patients and healthy donors. Using UMAP for dimensionality reduction, the study reveals increased heterogeneity and dispersion of T cell populations, as well as significant variability in marker expression in sepsis, underscoring the complexity of immune responses. Reproduced with permission from Ref. [104] © 2024 American Chemical Society.

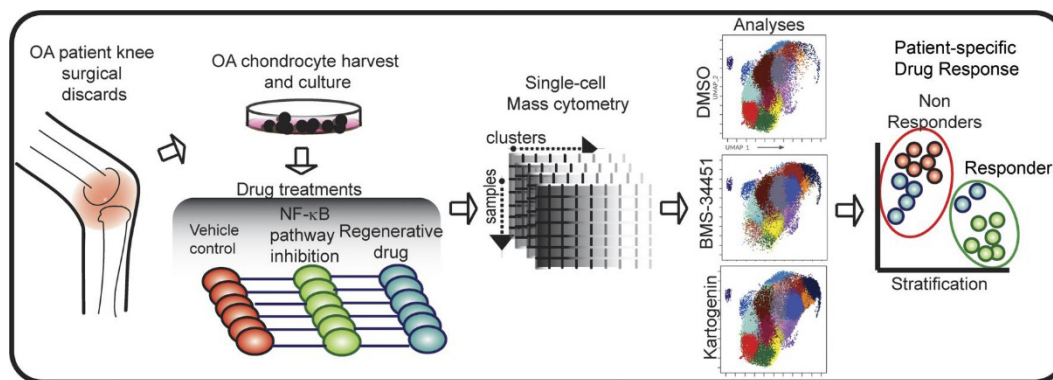


Fig. 12 A single-cell platform based on CyTOF for analyzing the effects of preclinical drugs on cartilage homeostasis. Osteoarthritis (OA) chondrocytes derived from knee replacement patients were treated with various drugs. CyTOF identified 25 distinct cell clusters. BMS-345541 uniformly reduced inflammation and senescence markers, whereas kartogenin exhibited limited effects with heterogeneous responses. This platform aids in patient stratification and drug screening. Reproduced from Ref. [106] © 2022 The Authors.

few as 10 gold nanoparticles (AuNPs) per cell, while simultaneously performing multiparametric phenotyping. Compared to traditional flow cytometry, mass cytometry better overcame autofluorescence issues in tissue samples. By evaluating the distribution of AuNPs with different surface coatings, they found that amphiphilic ligand-coated AuNPs effectively targeted myeloid dendritic cells in lymph nodes. These nanoparticles were subsequently used to deliver peptide antigens, significantly enhancing the efficacy of a vaccine in a melanoma mouse model. This research highlights the potential of mass cytometry to elucidate the *in vivo* behavior of nanoparticles and to inform the design of nanoparticle-based therapies.

Challenges and Prospects

The integration of metal isotope-tagged nanomaterials into mass cytometry and IMC represents a significant advancement in biomolecular profiling, providing deep insights into complex biological systems. However, several challenges must be addressed to fully realize the potential of these technologies in both clinical and research settings:

(1) **Biocompatibility and cytotoxicity control:** Although many metal isotopes are stable, they may exhibit cytotoxic effects at certain concentrations or upon cellular internalization. Developing nanomaterials that are effective for tagging while remaining non-toxic requires comprehensive *in vitro* and *in vivo* testing to evaluate their long-term effects on cellular health.

(2) **Clinical translation barriers:** Moving from research to clinical application involves challenges such as cost competitiveness compared to existing fluorescent labeling technologies. The synthesis of rare-earth metal isotopes (e.g., lanthanides) and nanoparticle functionalization currently incur higher costs, which need optimization for large-scale clinical deployment. Additionally, standardizing quality control procedures is essential; inconsistencies in nanomaterial synthesis such as size distribution and surface charge, can lead to batch variability in both non-spatial and spatial analyses. The necessitates establishing GMP-compliant manufacturing protocols and regulatory frameworks.

(3) **Lack of standardized protocols:** Variability in synthesis, functionalization, and application of metal isotope-tagged nanomaterials results in performance inconsistencies across laboratories. Developing unified guidelines for quality assurance—such as specifying particle size CV (< 5%), zeta potential ranges, and metal loading efficiency—is crucial for reproducibility and clinical validation.

(4) **High-dimensional data analysis:** The complexity of mass cytometry and IMC datasets demands advanced computational tools. While artificial intelligence (AI) and machine learning (ML) offers solutions, creating robust models capable of extracting biological insights from multi-omics integration remains a challenge.

(5) **Multi-omics data integration:** Combining mass cytometry findings with genomic, transcriptomic, and metabolomic data is vital for personalized medicine, yet technical and analytical barriers hinder seamless

integration.

Despite these challenges, metal isotope tags are pivotal for advancing mass cytometry and IMC. Future developments include:

(1) Material innovation: Designing next-generation metal isotope-tagged materials with enhanced stability, biocompatibility, and multifunctionality (e.g., dual diagnostic-therapeutic agents). These improvements aim to reduce non-specific binding and improve signal-to-noise ratios, surpassing traditional fluorescent labels in sensitivity.

(2) Liquid biopsy applications: Compared to tissue analysis, metal isotope-tagged nanomaterials offer advantages in liquid biopsy, such as minimally invasive sampling, real-time monitoring of circulating tumor cells (CTCs), and early detection of treatment resistance. The high multiplexing capacity (>100 markers) enables profiling of CTC heterogeneity at single-cell resolution, overcoming spatial limitations inherent to tissue sampling. Preclinical studies could explore their utility in tracking metastatic signatures or drug response dynamics in blood samples.

(3) AI-driven optimization: Machine learning algorithms can streamline nanomaterial design, such as predicting optimal isotope binding kinetics, and automate data deconvolution, accelerating biomarker discovery in precision oncology.

(4) Emerging applications: As mass cytometry and IMC capabilities expand, new applications are anticipated regenerative medicine, microbiome research, and environmental health. The multifunctionality of metal isotope-tagged nanomaterials positions them as valuable tools in these areas, fostering innovative research and therapeutic strategies.

In conclusion, although challenges related to cost, quality control, and clinical validation persist, interdisciplinary collaboration among chemists, biologists and engineers will be essential to unlocking the transformative potential of metal isotope-tagged nanomaterials for biomedical research and diagnostics.

CRedit Author Statement

Dongling Jia and **Minhui Cui**: investigation and writing original draft. **Weiwei Wang**: formal

analysis. **Jielin Sun**: conceptualization and supervision. **Xiaoxiang Chen**: conceptualization, supervision, writing–review, and editing.

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Conflict of Interests

The authors declare no conflict of interest.

Ethical Approvals

There are no ethical concerns in this study.

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