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Perspective

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Deep 3D histology powered by tissue clearing, omics and AI

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To comprehensively understand tissue and organism physiology and pathophysiology, it is essential to create complete three-dimensional (3D) cellular maps. These maps require structural data, such as the 3D configuration and positioning of tissues and cells, and molecular data on the constitution of each cell, spanning from the DNA sequence to protein expression. While single-cell transcriptomics is illuminating the cellular and molecular diversity across species and tissues, the 3D spatial context of these molecular data is often overlooked. Here, I discuss emerging 3D tissue histology techniques that add the missing third spatial dimension to biomedical research. Through innovations in tissue-clearing chemistry, labeling and volumetric imaging that enhance 3D reconstructions and their synergy with molecular techniques, these technologies will provide detailed blueprints of entire organs or organisms at the cellular level. Machine learning, especially deep learning, will be essential for extracting meaningful insights from the vast data. Further development of integrated structural, molecular and computational methods will unlock the full potential of next-generation 3D histology.

Histology has been used to examine the microanatomy of tissues at the cell level and to link tissue structure to function for more than two centuries[1]. Traditional histology requires sectioning tissues to generate thin, transparent slices from otherwise opaque biological tissues, which then allows imaging cellular and molecular details. Yet, serial sectioning and imaging are impractical for larger organs or whole rodent bodies, which means that scientists have had to choose samples based on preconceived ideas, leading to confirmation bias and limiting the potential for discovering unanticipated mechanisms and phenomena.

The problem is most apparent at the whole-organism level when studying interconnected systems of multiple or all organs. Therefore, new approaches for assessing the whole specimen could lead to a fundamental shift away from biased (that is, focused on pre-selected tissues and their molecules) to unbiased assessment of biological processes. The power of such holistic approaches has already been shown in many studies in lower organisms[2,3]. To bring the same level of analysis to mice, humans and other opaque higher organisms, tissue-clearing approaches that enable 3D imaging of intact biological specimens at the cellular and subcellular level have emerged as promising technologies. Combining tissue clearing with omics approaches and deep learning analysis will further accelerate the study of biological systems.

A prime example of a process that spans multiple scales is cancer development and cancer metastasis. Cancer is both a local and a body-wide phenomenon[4]. To fully understand the whole metastatic cascade and to develop metastasis-specific treatments, we need tools to find and characterize individual cancer cells in the context of the whole body. This will allow us to characterize their molecular diversity and to assess the efficacy of drugs (that is, their targeting and the induction of desired effects) down to the single-cell level in every part of preclinical animal models and human samples.

Not only cancer but most diseases also have a systemic component, just as normal physiology and development do. Also, next-generation treatment modalities such as engineered T cells[5], somatic gene editing[6] and regenerative medicines such as stem cells[7] and engineered tissues[8] (including organoids[9]) all require accuracy and control at the cellular level to be truly effective and must be

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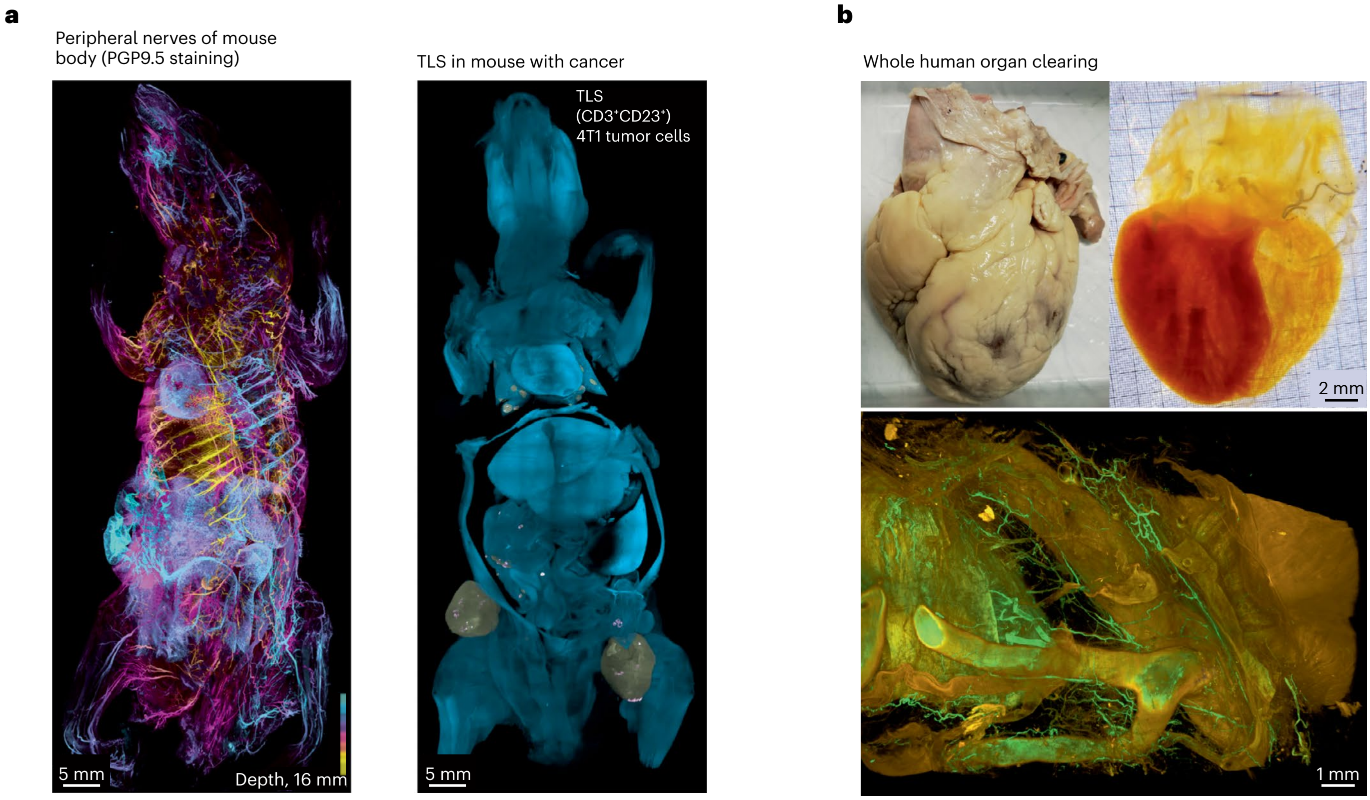

Fig. 1 | Optical clearing enables imaging of whole mouse bodies and human organs. a, Imaging of optically transparent whole mice stained with antibodies specific to the peripheral nerve marker PGP9.5, also known as ubiquitin carboxy-terminal hydrolase L1 (left) to reveal the complexity of the nervous system (physiological state, 4 weeks old) or markers ($CD3^+CD23^+$) for tertiary lymphoid structures (TLS) (right) to reveal these in a mouse with cancer. Adapted from ref. 27, CCBY 4.0. **b**, Whole human organs, such as the heart, can be made optically transparent, stained and imaged using light-sheet microscopy. Bottom, dextran-labeled vessels are shown in green, and plaques as autofluorescence are shown in gold. Adapted with permission from ref. 61, Elsevier).

assessed at a holistic level. Clinical pathology would also benefit from a more complete characterization of tumors and tissue samples, as is already being explored in early clinical investigations using tissue-clearing techniques for tumor biopsies[10,11].

In addition to the challenges caused by tissue selection, the analysis of imaging data can introduce additional biases. For example, filter-based data analysis in commercial visualization software requires human input to define thresholds, which is largely subjective. Optimal image analysis pipelines, however, aim to be as independent of human subjectivity as possible to limit interobserver and intra-observer variability. Recent developments in machine learning-based image analysis are now bringing us closer to this goal. Although these approaches require further development to achieve full objectivity, that is, to operate independently of human-generated training data, deep learning methods already surpass humans in many data analysis tasks[12].

In sum, biomedical research needs to evolve beyond the analysis of structural and molecular biology in selected tissue sections, expanding its focus to entire organs and organisms. Here, I will outline a path for combining unbiased volumetric imaging with unbiased molecular profiling of biological samples, a pursuit that I consider both a major goal and a grand challenge in biomedical research. Finally, this Perspective will discuss recent advances in deep learning approaches aimed at the unbiased and integrated analysis of large-scale imaging and molecular datasets.

Deep imaging of whole organisms using optical tissue clearing in 3D

The inability of classical histology to preserve the 3D spatial context has spurred the development of optical tissue-clearing techniques that enable the imaging of intact specimens with cellular or subcellular resolution (Fig. 1)[13]. When combined with light-sheet microscopy, tissue clearing enables the investigation of samples ranging in size from whole mouse and human embryos to entire mouse bodies and human organs at the cellular level[14–17]. Ongoing refinements of clearing and labeling methods aim to optimize results across diverse organs and tissues.

In recent years, we have seen diverse applications of tissue clearing in biological research. For example, in mice, tissue clearing has helped to identify brain regions regulating feeding[18], parental behavior[19] and mood and anxiety[20] as well as dopaminergic neuronal circuits in the substantia nigra[21] and cortical brain regions downstream of whisker-evoked sensory processing[19]. In the mouse gut, tissue clearing has revealed interactions between the peripheral nervous system and local macrophages[22] while tissue clearing has shed light on the morphogenesis of human exocrine glands and neurovascular and skeletomuscular structures in the developing human head[23].

To make tissue-clearing methods widely usable, it is essential to develop more standardized protocols. While we have witnessed the continuous development of various tissue-clearing methods, they are mostly tailored for specific uses. Similar to the user-friendly single-cell RNA sequencing (scRNA-seq) technologies, such as the 10x platform, that have revolutionized single-cell biology, standardizing protocols and instruments will enhance the accessibility of tissue-clearing methods.

I foresee that tissue clearing and labeling will become increasingly automated to standardize and accelerate laborious sample processing. Meanwhile, making open-source light-sheet microscopes available for large-scale 3D imaging will help to democratize the imaging of cleared tissues. We also need easy-to-use software tailored for common

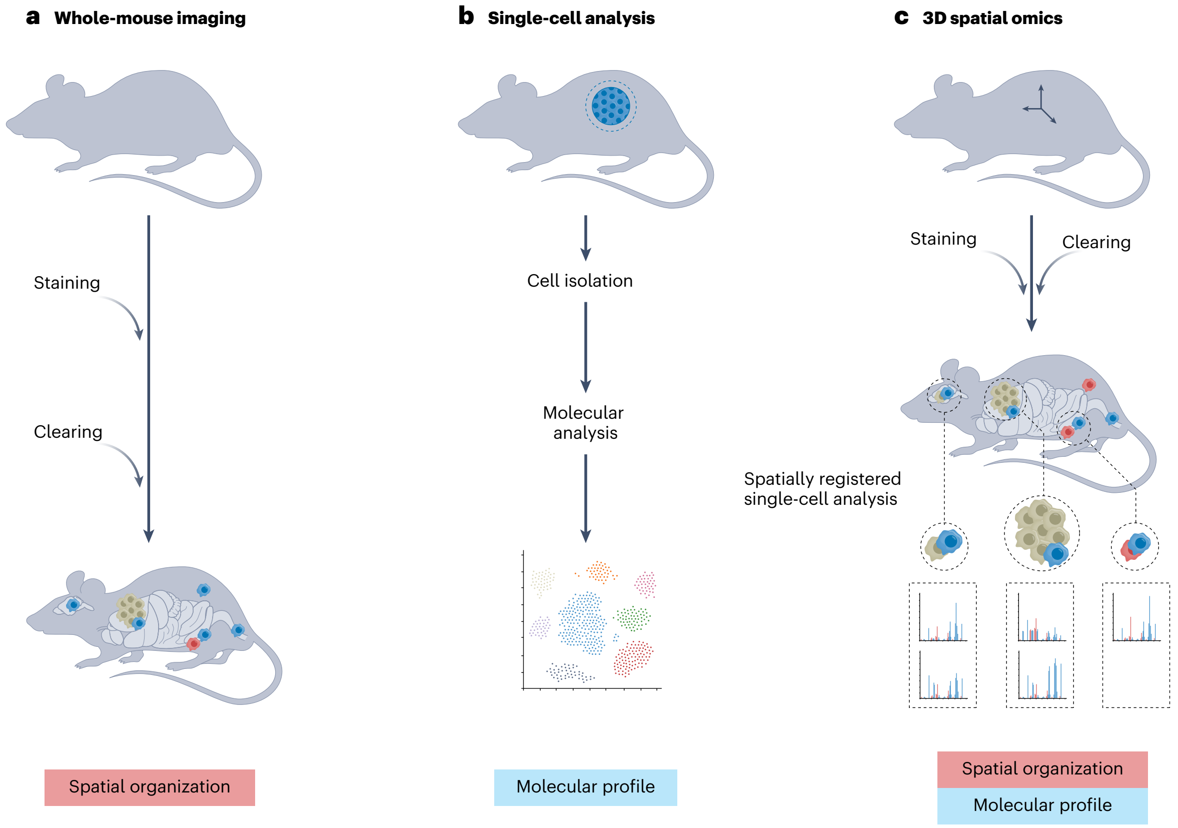

Fig. 2 | Three-dimensional omics for spatial molecular maps. a, Current imaging methods can provide whole-body maps at cellular resolution but only for a few markers. **b**, scRNA-seq and single-cell proteomics can provide high-sensitivity analysis of individual cells from tissues but lose spatial context. *t*-SNE, *t*-distributed stochastic neighbor embedding. **c**, Future 3D spatial omics technologies will provide whole-body maps of the distribution of biomolecules in whole animal bodies.

analysis tasks, yet flexible enough to allow optimizations for diverse applications. Unlike scRNA-seq, imaging data vary substantially in terms of resolution, complexity and labels, requiring tailored analysis methods, now primarily based on deep learning, for segmentation and classification. Thus, while the standardization of basic procedures and analyses will ease access and ensure data comparability across groups, retaining flexibility in both clearing and analysis protocols is crucial.

Imaging more specifically

Labeling specific cells and molecules in whole specimens has represented a major bottleneck for tissue-clearing applications. Small-molecule dyes, while extensively used in many clearing protocols, can only stain a limited range of biomolecules. Methods such as SWITCH[24], CLARITY[25] and immunolabeling-enabled 3D imaging of solvent-cleared organs (iDISCO)[26] have enabled the use of antibodies in mouse organs. We recently developed wildDISCO[27], a method that enables the use of full-length antibodies for staining entire mouse bodies, thus making the full arsenal of thousands of commercial antibodies available for whole-organism studies (Fig. 2a). RNA and DNA molecules can be labeled and imaged in some clearing protocols; however, these are currently challenging to apply to large specimens on a routine basis[15,28–30].

The limitations of labeling methods can be partially overcome by combining them with unbiased molecular profiling (omics) methods (Imaging more, faster and smarter). I expect that future developments will introduce new methods to increase the number of detectable molecules, for example, through 3D in situ sequencing in cleared tissues or by substantially enhancing our ability for multiplex labeling in cleared tissue. Furthermore, there is a pressing need to develop new technologies for antibody labeling of very large specimens, such as human organs.

The extended periods required to complete current labeling protocols, ranging from many days to weeks, represent another bottleneck for the widespread application of unbiased 3D imaging. Substantial efforts are needed to develop engineering solutions capable of achieving large-specimen labeling within minutes to hours, akin to the increases in clearing speed already achieved for small samples[31,32]. Through these advancements, we will be able to image increasingly larger and, therefore, more intact biological samples.

Imaging more, faster and smarter

Imaging labeled cells and molecules in large, cleared tissues involves a tradeoff between resolution and the size of tissues that can be imaged. Objectives with higher numerical apertures (NA) tend to have a shorter working distance, thereby reducing the maximum depth that can be accessed in a tissue. Simultaneously, there is a general inverse relationship between field of view and NA, necessitating more images to cover the same volume at a higher resolution, thus reducing overall imaging speed. Although various imaging modalities have been employed for cleared samples, each affected by tradeoffs among different imaging parameters, light-sheet microscopy has emerged as the most effective, delivering reasonable resolution (~1–2 μm in *xy* and ~5–10 μm in *z* dimensions) within a reasonable timeframe[33].

High-speed and high-resolution light-sheet microscopy methods, such as lattice light-sheet microscopy, have been developed to track individual cells in 3D. However, these approaches are not applicable to cleared samples that are several centimeters in size, due to the short working distance of the required high-NA objectives. Millimeter-sized specimens can be imaged at submicrometer resolution using approaches such as axially swept[33] or tiling scan[34] light-sheet microscopy. Even subdiffraction (~60–90 nm) resolutions are achievable by combining expansion microscopy with lattice light-sheet microscopy[35].

Creating submicrometer-thick light sheets with centimeter-scale lengths (or scannable over centimeters) and high-NA objectives with centimeters of working distance would substantially enhance the capabilities of light-sheet microscopy. However, substantial physical and engineering limits remain to be overcome. Recent adaptations of innovations from the electronics industry to the life sciences, as pioneered in the ExA-SPIM project, have demonstrated that substantial progress in field of view and working distance is possible[36].

A related limitation involves the extensive scan time required for imaging large samples. Currently, data collection for an entire adult mouse body can require 4 d to 2 weeks of continuous scanning. Ideally, this process should be completed within a matter of hours. Accelerating scanning will hinge on advances in both hardware and software development specific to light-sheet microscopy. For example, the development of imaging lenses with larger fields of view and working distances, combined with methods to create longer, uniform light sheets[37], either physically or through digitally scanned light sheets, and employing axial sweeping[33] or tiling scan modes[34,38] will be critical for mapping larger human organs, including the heart, the kidney and the brain at the cellular level. Additionally, developing methods to parallelize image acquisition by using multiple light sheets simultaneously could substantially increase the speed of data acquisition[39]. The integration of machine learning into scanning systems, as discussed below, has the potential to increase the quality of scans and to decrease scan time and data size.

Very large specimens, such as whole human organs, pose additional challenges due to the limited working distance of the detection objective and quick deterioration of the light sheet within the sample. Currently, the most practical solution for imaging very large samples involves cutting these samples into pieces compatible with a given imaging setup. Alternatively, using consecutive centimeter-thick tissue slices ('slabs') allows for imaging with tilted light-sheet geometries (such as inverted selective plane illumination microscopy[40] or light-sheet microscopy[41]) or with oblique plane light-sheet microscopes[42], which permit scanning large samples with arbitrarily large lateral dimensions (albeit with limited thickness) without sacrificing resolution. These approaches render the organ-scale imaging problem tractable, enabling the imaging of human organs such as the kidney[17], albeit at the cost of minor slicing artifacts and increased computational complexity for slice registration.

Human involvement is necessarily minimal during long-term imaging. However, the changing optical properties and information content of the sample ideally require adjustments during automated image acquisition to maximize image quality and to minimize scanning time. The ideal solution involves designing smart microscopes that perform on-the-fly data analysis and automatically adjust microscope and scanning parameters while samples are being imaged. This approach can (1) skip image planes lacking valuable information, such as blank tiles, (2) provide optimal light-sheet illumination based on the signal level at each depth, (3) adjust additional microscope settings, such as light-sheet thickness and camera exposure to optimize the signal-to-noise ratio throughout the imaging process, (4) identify and report on the quality of labeling and imaging during the process to assess whether it is worth scanning the entire sample and (5) learn from previous scans to optimize future scans of similar samples.

Initial efforts in various imaging modalities have been directed toward, for example, performing automated adaptive alignment, compensating for changing sample distortions and growth, improving autofocusing[38,43–48] or conducting on-the-fly basic analysis of image content[49–51]. However, to design truly 'smart' imaging systems that substantially increase the efficiency of large-tissue imaging, it will be essential to integrate machine learning into the microscope controls[52].

What is on the horizon for cleared-tissue imaging

Substantial challenges remain in fully unlocking the potential of tissue clearing. A key challenge is the limited multiplexing capability beyond the few targets currently feasible. Broad fluorophore spectra, strong blue–green autofluorescence and the limited spectral region available with current instruments and labels limit the number of colors that can be used in a single scan. Expanding the usable spectral range into the near-infrared (800–1,100 nm) or long-wavelength (1,200–1,600 nm) windows could alleviate this crowding. Adding fluorescence lifetime contrast could also substantially boost resolvable labels[53]. Alternatively, intrinsically narrow-band Raman microscopy offers multiplexing capabilities[54], albeit with more complex instrumentation and currently lower signal-to-noise ratios limiting its adoption. In terms of labeling, multi-round antibody or RNA staining and in situ sequencing, well established for cells and small tissues[55–57] and already implemented in individual rodent organs for immunolabeling[24], could substantially increase the number of targets imaged in whole organisms.

Regarding instrumentation, improved detectors and higher data transfer speeds will enhance acquisition rates while maintaining high signal-to-noise ratios. Simultaneously, as expression level variations across tissues stretch the systems' dynamic range, smart microscopes, new signal-amplification strategies and improved detector technologies will be critical for realizing the full potential of multidimensional 3D imaging.

Deep molecular profiling in 3D

Historically, whole-tissue imaging and large-scale molecular profiling have been distinct domains. Single-cell transcriptomics and proteomics enable the assessment of a cell's molecular identity but necessitate tissue dissociation and a biased selection of tissues, thereby losing morphology and the broader context (Fig. 2b). This limitation restricts our understanding of the connection between tissue structure and cell function. Additionally, rare cells, which are often critical for physiology, can be lost among the multitude of other cells.

Similarly, early pathological changes often originate in individual cells that are undetectable against the tissue background. Identifying and profiling the initial cellular stages of disease could enable presymptomatic diagnosis and treatment. Currently, tissue sectioning for various spatial omics methods is the most common approach for isolating regions of interest identified in imaging data. A second-level isolation can be achieved using laser capture microscopy to obtain even single cells for transcriptomics[58] and, more recently, proteomics[59,60]. However, the critical loci for many diseases, such as micrometastases or initial Alzheimer's plaques, occupy only a tiny fraction of the total tissue volume, and their exact locations are unknown a priori, making it challenging to identify the optimal regions for further analysis.

Tissue clearing of whole specimens can help overcome this bottleneck by providing the cellular resolution necessary to identify even the rarest cell types and the smallest pathological changes, but performing molecular profiling after extensive clearing procedures presents challenges. However, we have demonstrated that proteomics analysis in DISCO-cleared tissues yields results indistinguishable from those of fresh tissue[61]. RNA preservation in some clearing techniques is sufficient to perform in situ hybridization in 3D tissues[15,28–30], including multiplexed imaging in brain samples[28,29].

In the future, methods to directly extract samples of interest from cleared tissues with high efficiency, similar to the robot-assisted extraction we have used for proteomics analysis (Fig. 3a)[61], or approaches for 3D in situ labeling (maybe similar to Light-Seq[62]) (Fig. 3b) might increase the throughput and multiplexing capability of molecular profiling in whole tissues (Fig. 2c).

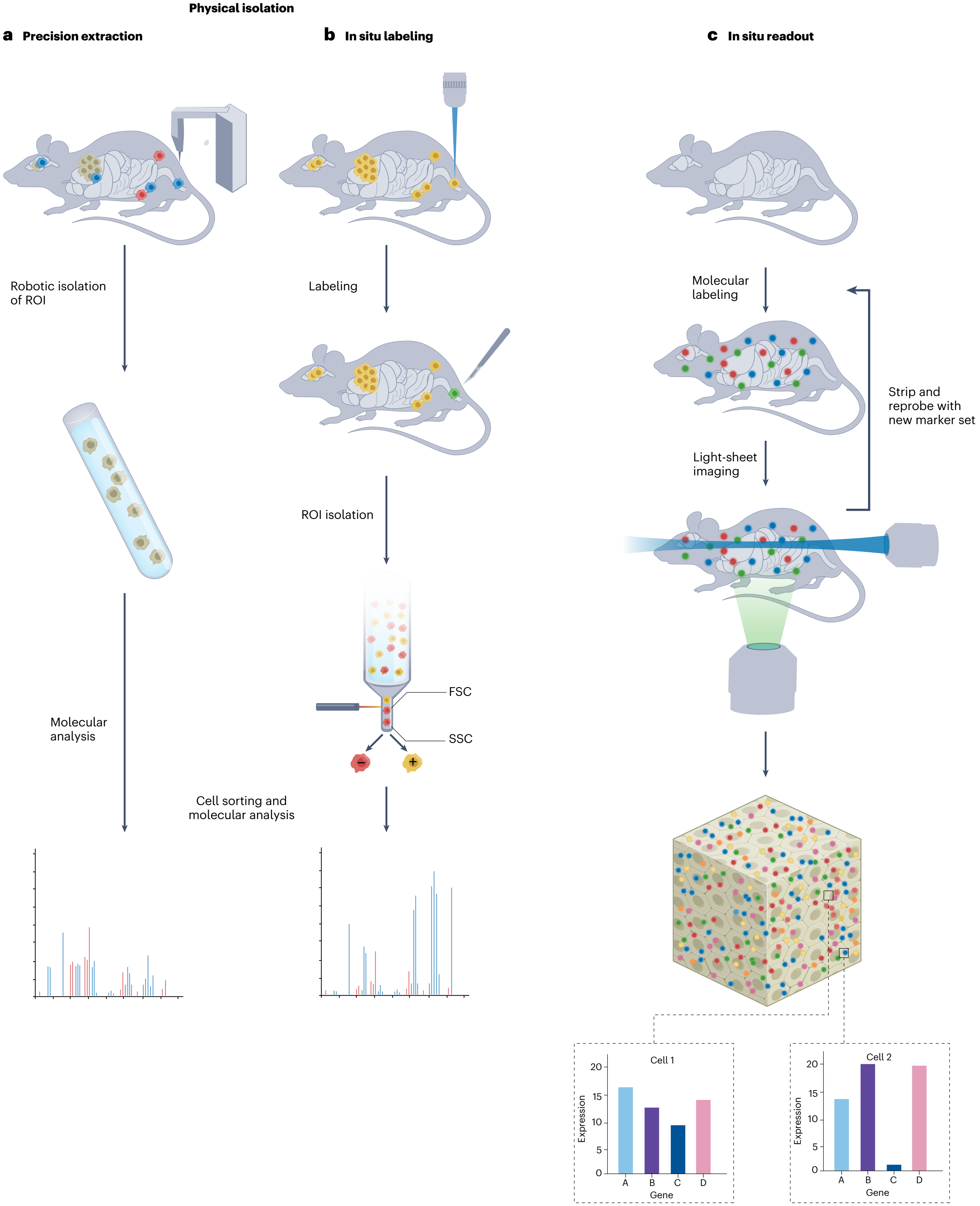

Fig. 3 | Conceptualizing possible approaches for 3D omics. a, Small samples can be physically extracted using minimally sized biopsies and automated procedures. Currently, samples can be as small as 60 cells[61]. ROI, region of interest. **b**, Building on current small-scale approaches, in the future, cells of interest in whole organisms can be labeled in situ, for example, using photochemistry, and then isolated using cell sorting. FSC, forward scatter; SSC, side scatter. **c**, In the future, we might be able to read out molecular information directly in the large volumes of whole organisms, building on current approaches such as seqFISH and MERFISH[55,56].

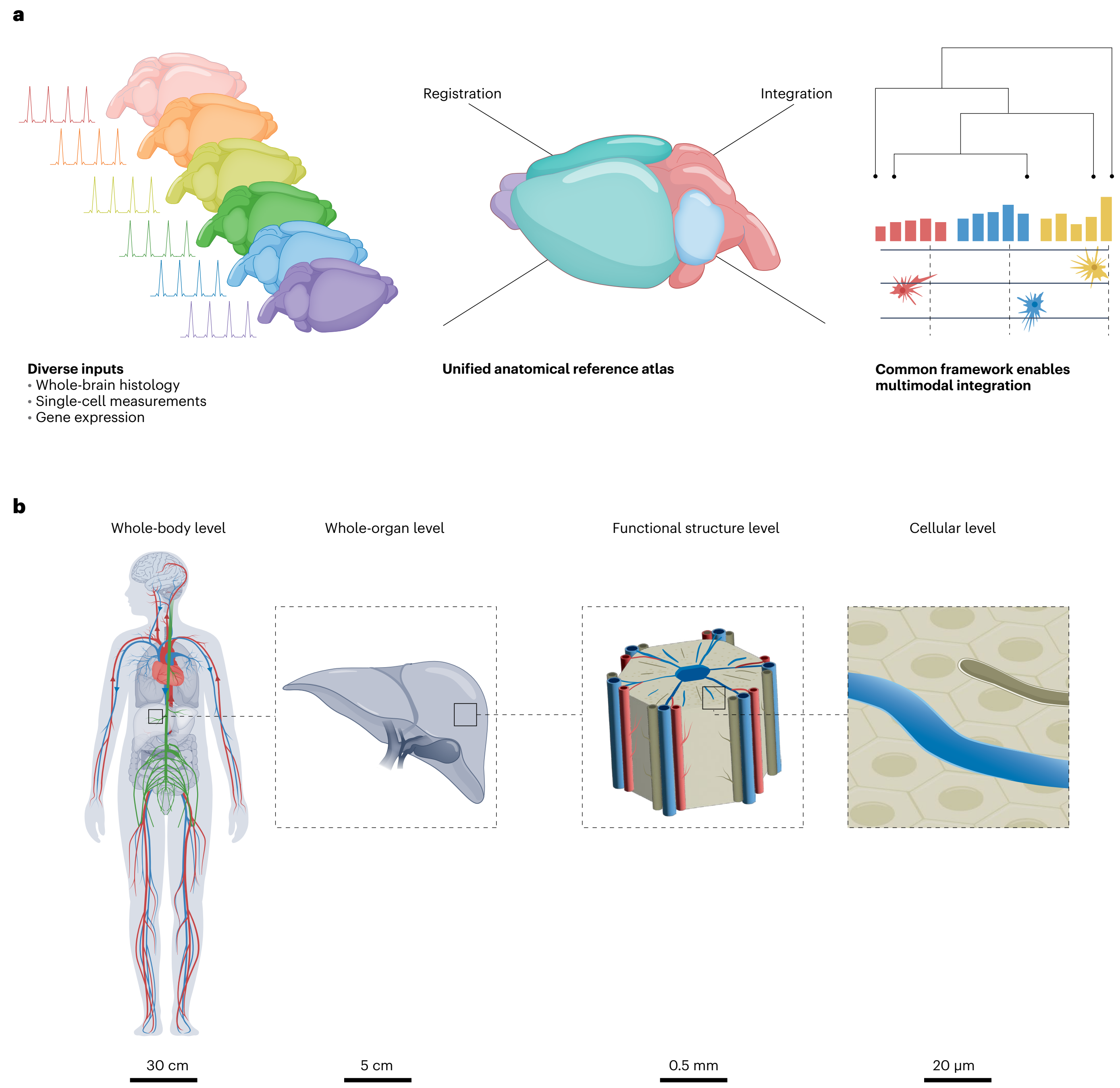

Fig. 4 | CCFs are essential for comparing and integrating different datasets. **a**, The Allen Brain Atlas as an example of a common coordinate and integration system: a common anatomical reference framework allows registration across individuals and modalities (whole-brain histology, single-cell physiology, multiomics). Common references, in turn, enable multimodal integration across data types and species. Center: adapted with permission from ref. 73, Elsevier. Right: reproduced from ref. 126, CCBY 4.0). **b**, A future body-wide CCF will need to span scales ranging from meters to nanometers to integrate all data that can be obtained using the full complement of methods in our arsenal. A hierarchy of CCFs might be necessary to accommodate all scales.

Spatial omics methods are increasingly being integrated with tissue imaging to combine molecular data with spatial context. These approaches include in situ sequencing, capture, barcoding and hybridization techniques, such as MERFISH (recently reviewed in ref. 63). Although these methods are currently slice based, they have yielded substantial insights, particularly in oncology and neuroscience[64–68]. However, sectioning introduces bias by limiting analysis to pre-selected regions, thereby constraining the ability to characterize heterogeneity and discover rare features. For instance, to fully reveal the molecular heterogeneity of micrometastases, it is essential to identify them throughout the entire organism rather than in just a few tissue slices, which at best represent less than 0.01% of the entire organism in a section-based experiment.

In principle, most spatial omics methods could be adapted to 3D cleared-tissue samples, enabling more comprehensive, unbiased and scalable 3D analyses.

Some potential future developments in spatial omics

Ultimately, our goal is to map every single cell analyzed with omics technologies back to its precise position in the 3D imaging data (Fig. 3c). This could, for example, be achieved through cell barcoding. In principle, barcoding every single cell within a sizable 3D volume for subsequent

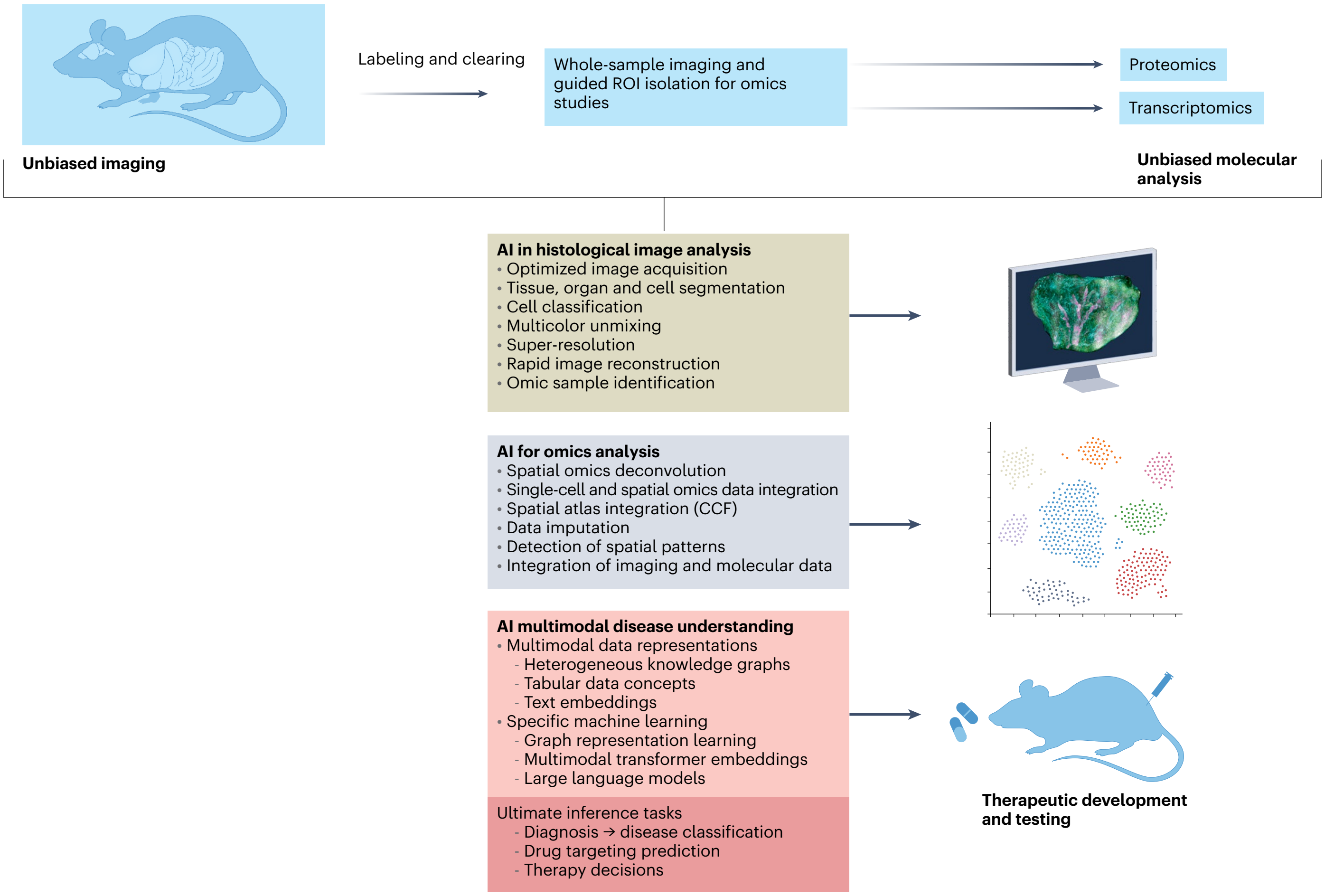

Fig. 5 | AI tools are essential at all stages of 3D omics analysis. The large size and multidimensional nature of the combined imaging and molecular data require computational tools starting from the optimization of image acquisition to image analysis, omics data integration and extraction of biological and medical insights. Monitor image reproduced with permission from ref. 17, Elsevier.

molecular analysis should be feasible. The barcode diversity required to label the approximately 300 million cells of a mouse liver[69] is within the capabilities of current barcoding approaches, requiring barcodes of at least 15 bases (more for efficient error correction). However, reading these barcodes in cleared tissue with single-cell precision remains beyond our current capabilities. Approaches similar to MERFISH or seqFISH could be adapted for 3D imaging, enabling a direct readout of molecular information from the imaging data[55,56]. Finally, imaging-based techniques could be complemented by advanced imaging-free molecular tomography methods[70].

All methods face immense detection range requirements, up to 12 orders of magnitude in the proteome, for example[71]. This poses substantial challenges for fully profiling heterogeneous samples. Advances in sensitivity, dynamic range and the extraction of signals from incomplete data will be crucial for resolving 3D molecular maps.

The ultimate goal is to develop dynamic 4D approaches that profile spatially mapped cells in living, transparent mammals over time. Such approaches could yield developmental and disease insights that are impossible to achieve with static tissue data.

Mapping molecular data to common reference frameworks

To enable comparability and integration of data from different experiments and groups, developing common coordinate frameworks (CCFs) for high-resolution molecular and spatial data is essential (Fig. 4)[72]. Existing CCFs, such as the Allen Brain Atlas, facilitate multimodal data registration, even across species[73]. CCFs can be static for stable structures, like the brain, or dynamic for variable systems, such as the vasculature. The design of CCFs is complicated by natural variations in organ size and shape across individuals, which can be influenced by factors such as age, sex, genetic background, environmental exposures or disease states. The requirement to span length scales from meters to nanometers further complicates matters. It remains debated whether a single CCF suffices across scales or whether a hierarchy of different models is necessary. Artificial intelligence (AI)-assisted mapping can already automatically register new data into reference atlases at the organ scale[74]. However, even approximate or relative cell locations would still offer valuable context (for example, proximity to vessels for cancer cells).

Ultimately, CCFs are envisioned to serve as open platforms for community-wide data integration and sharing. Initiatives such as the Human Cell Atlas underscore the importance of seamlessly integrating spatial details with community data as key objectives for the future[75]. Dedicated repositories for spatial and single-cell data have deepened insights by facilitating integrated analyses. Expanding this open-science concept to include next-generation molecular anatomy maps will further propel discovery.

Deep learning for large data analysis in 3D

In the previous sections, I discussed how rapid developments in experimental techniques enable the unbiased investigation of biological processes, which presents an important step toward removing human biases from sample and marker selection, enabling hypothesis-free discovery research.

The next step toward creating unbiased and scalable 3D molecular atlases involves the development of robust algorithms. These algorithms must be capable of not only analyzing imaging and molecular data separately but also integrating them to generate biological insights (Fig. 5). In terms of image analysis, computer vision algorithms have automated many time-consuming steps, while sophisticated post-processing pipelines have substantially improved data quality. Simultaneously, increasingly sophisticated algorithms are extracting the most meaningful data from various omics[76,77] and imaging[78,79] data types.

Recent advances in self-supervised and unsupervised learning, exemplified by algorithms such as Gemini (Google Bard) and ChatGPT (OpenAI) in natural language processing, in image and video generation with tools such as Midjourney, Dall-E and Sora, along with Segment Anything in image analysis, have outpaced traditional methods. Self-supervised and unsupervised learning approaches are especially pertinent for histological data, characterized by its abundance of unannotated datasets, including the vast number of histopathological images generated daily in clinics. A prime example of a potent self-supervised algorithm is contrastive learning, which learns similarities and dissimilarities between images. This approach promises to deliver efficient methods for tasks such as cell phenotype classification, segmentation and disease classification[80]. For instance, a contrastive learning algorithm might learn to recognize various features of cell morphology, such as shape, size and texture, by training on segments of both pathological and healthy histology images. This internal representation of features can then be fine-tuned with a smaller set of pixel-annotated slides, accurately segmenting cancerous cells and thereby enhancing diagnostic accuracy.

Deep learning for image analysis

Algorithm development for a wide array of challenges in biological imaging is now predominantly guided by supervised deep learning approaches. In the realm of next-generation histology, several areas have been identified where deep learning-based methods and other machine learning techniques are poised to make substantial breakthroughs. Conceptually, AI applications can enhance image quality, segment distinct entities within images (such as cells, subcellular organelles or tumors) and classify various objects (including organs and cell types) contained in the images. These applications have been comprehensively reviewed recently[78], and I will be highlighting the aspects most relevant to large-scale imaging endeavors.

Deep learning for improving image quality and imaging speed

Gathering a full imaging dataset for a complete mouse or human organ can take from a few days to 2 weeks, depending on the desired resolution and the number of imaging channels. Approximately half of this period is consumed by manual adjustments in the light-sheet microscope, such as filter changes, focus realignments, corrections for color distortions and moving the specimen. These procedures could be streamlined by implementing the ‘smart microscope’ strategy described above, which automates many of these adjustments. The rest of the scanning time involves illuminating the sample and capturing images. Using algorithmic improvements to enhance image quality has the potential to shorten exposure times, substantially cutting down the total time required to gather comprehensive datasets. Moreover, robust computational techniques for the efficient separation of colors in multicolor 3D images after acquisition (without manual filter changes) would further decrease delays associated with microscope adjustments.

Reconstruction of images from noisy data has received substantial attention in recent years, mainly for live cell and neuroscience applications[81–83], but these techniques could also play a pivotal role in reducing exposure times for 3D histology applications. In the best scenarios, signal-to-noise ratios can be increased by up to 60-fold, dramatically reducing the time required for image acquisition. Interestingly, in certain situations, even high-quality ground truth datasets are not necessary for training denoising neural networks[84]. Yet, specialized methods specifically developed and tested for large-scale 3D imaging data have not been widely reported.

The current state-of-the-art for acquiring high-quality multicolor images involves changing filters for each imaging plane individually, capturing all colors one after the other before proceeding to the next plane. This is due to the inevitable drift in both the sample and the microscope over extended imaging sessions spanning several days. Although this method is very time consuming, it makes it easier to combine the different channels. AI solutions could potentially address the challenge of aligning these channels and enable the simultaneous capture of multiple colors, greatly speeding up the process of gathering image stacks. Despite the seemingly simple concept, applying linear unmixing[85] and phasor[86] approaches faces substantial challenges due to the complexity of capturing multicolor images in large specimens. An AI-based approach might offer a new way to overcome these obstacles.

Deep learning for image classification, segmentation and augmentation

To understand the immense amounts of imaging data, it is crucial to differentiate objects from the background and to classify these objects into functional classes (for example, organs, cells, subcellular compartments). Image segmentation and classification have been central challenges in image analysis, spurring the deep learning revolution in computer vision[87]. Segmentation addresses a pixel-level classification challenge. Early research in biology focused on segmenting cells and subcellular compartments, achieving success across various datasets[88–90]. In many instances, using these networks necessitates substantial adaptation to meet specific user needs. By contrast, nnU-Net is a flexible segmentation network that automates its configuration process, including the optimization of preprocessing, network architecture, training and post-processing for each new image analysis task, and delivers results comparable to those of specifically optimized methods[91].

Deep learning can also be applied to the segmentation of larger objects. For example, we and others have applied deep learning to identify metastases[92,93], to reconstruct the whole mouse brain vasculature[94,95] or to segment organs in cleared mice[74]. Recently, unsupervised approaches such as contrastive learning have shown promise for image analysis in more complex applications, for example, for discriminating cell phenotypes in microscopy images[80]. For this, practical optimization functions, which can provide specific shape priors for the biological objects (such as connectivity and structure homology), are important[96,97].

Deep learning can also reveal information beyond what the human eye can see. The concept of ‘virtual staining’ or ‘augmented microscopy’ has proven powerful in generating histological stains from autofluorescence or other label-free data and the transformation of one stain into another (recently reviewed in ref. 98). Virtual staining was able to generate virtual immunostainings from other imaging data to label subcellular compartments from autofluorescence of cultured cells[99] or to reconstruct various diagnostic immunohistochemistry stains for cancer diagnosis from simple H&E stains[100]. Multiplexed immunofluorescence data can also be generated from individual channels in clinical histological slides[101]. However, the methods have yet to be generalized to a 3D histology setting, with few exceptions such as the generation of virtual cytokeratin 8 staining from H&E stains in human prostate cancer samples[11] or basic label-free reconstruction of tissue architecture[102]. More work needs to be done to generate high-quality training data and to identify the best use cases for augmented microscopy in 3D histology, as even basic H&E staining, 3D imaging and AI-based analysis of prostate cancer samples has recently been shown to increase the predictive power of histopathology[103].

Generation of 3D ground truth data, that is, labeling and categorizing imaging and molecular data by experts for training deep learning-based approaches, remains a major bottleneck. Thousands, if not millions, of such labels are typically required to train deep neural networks. While innovations such as the use of virtual reality visualization[104] can speed up image annotation in 3D, it is still a laborious and error-prone process. Community efforts to generate more high-quality, interlaboratory, 3D datasets with cell-level resolution would be of substantial value for algorithm developers, as they have been for two-dimensional (2D) approaches and medical image analysis[91,105]. Also, increasing the participation of the wider community might increase the amount of available training data, for example, by gamifying image annotation[106]. To ensure comparability of results from different laboratories, it will also be essential to define quality metrics for human image annotation data.

As the power of supervised algorithms depends heavily on the size of the ground truth data, we also need unsupervised, human-free approaches for ground truth generation.

Clever experimental strategies or methods to generate synthetic training data might also be able to provide substantial amounts of ground truth data with minimal human post-processing involvement.

Deep learning for imaging and molecular data integration

To achieve a holistic, system-wide description of the entire sample and its pathologies, deep learning methods that integrate data from multiple imaging and molecular modalities will be needed to identify spatial and molecular features and classify them according to their physiological or pathological function.

One major aspect is to identify a unified data representation to combine all data sources for a machine learning model. The recent success of LLMs has shown that text representations are very powerful for many tasks. Still, the information loss when representing images as text or, alternatively, as tabular data is immense. Therefore, I believe that a dynamic representation that efficiently embeds pixel information is required for digital histology. Highly promising as representations of multimodal datasets are heterogeneous and knowledge graphs, which can integrate different data types and allow the application of highly powerful and explainable graph neural networks[107–109]. Importantly, this multimodal data integration will allow a single deep learning model to make overall predictions, for example, to identify and classify a cancer lesion in a histological sample.

A major analytical challenge will be the analysis of 3D molecular data and their integration with 3D imaging data. Many of the principal problems are also encountered by the current generation of 2D spatial omics methods. Similar to these approaches, I expect that the initial 3D omics methods will either have a low spatial resolution (as our DISCO-MS method[61]) or will have low transcriptome and proteome coverage.

Low spatial resolution can be partially overcome by integrating spatial data with single-cell reference data that allow deconvolution of the expression profile obtained from the many cells in the experimental volume into cell type compositions. These methods are relatively advanced for 2D spatial transcriptomics data, where methods such as cell2location[110], SpatialDWLS[111] and RCTD[112] reach high accuracy in predicting cell compositions. I expect that these or similar methods will be applicable to 3D transcriptomic data with minor modifications. Microscopy data can also be directly used to increase the resolution of spatial methods using deep learning[113] and advanced statistical methods[114]. Similar methods have not been reported for proteomics data, where single-cell experiments are still in their infancy.

Low molecular coverage can be addressed by the imputation of missing values, which relies on expression profiles determined experimentally. For 2D data, several methods have been suggested. These either use scRNA-seq data references by themselves[115] or combine them with spatial correlations and imaging data[114,116]. Adapting these methods to 3D spatial transcriptomics data, especially when incorporating 3D spatial correlations and microscopy data, will likely necessitate substantial optimizations. In proteomics, imputation methods for bulk samples[117] are commonly used and are now being extended to single-cell experiments[118]. Further analysis tasks, such as identifying genes that vary across space and exploring how cells communicate, have been pursued in 2D contexts (reviewed in depth in ref. 77). Recently, graph-based algorithms have become a powerful tool for analyzing spatial data[116,119,120].

The quality of deep learning algorithms critically depends on the training data, and biases in the training data can be amplified by AI[121]. Therefore, it is essential to continuously work on developing methods to validate AI outputs and to ensure that training data selection is as varied and representative as possible. This includes covering the full range of data, from categories with a lot of examples to those with very few. Additionally, making deep learning models understandable and interpretable remains a challenge. Despite substantial research efforts[122], complete solutions for the increasingly complex models are still lacking. Improving explainability is critical for ensuring that researchers and clinical experts can trust and effectively use AI-generated insights.

Outlook and conclusions

The ultimate goal of biomedical research is to measure or accurately predict the molecular and structural identity of every single cell in an organism at any time. Transcriptomics and proteomics information from isolated cells has already expanded our views on how diversity in biological systems governs biological functions. In parallel, tissue-clearing-based 3D imaging of intact biological specimens at the cell level has started to reveal the true integrated anatomy of biological systems. In the near future, we will witness an accelerated merging of these two realms by achieving cellular-, even subcellular-level structural and molecular profiling in the same intact biological specimens.

Expanding tissue clearing to the fourth dimension, that is, time, by live transparency could add another layer of information. However, most of the major animal models such as rodents are not transparent. While rendering a living mouse entirely transparent is probably out of reach, achieving even partial transparency would already represent enormous progress. Chemical approaches, the basis of current tissue-clearing methods, are unlikely to succeed in living organisms as most of the chemicals used are toxic, but a genetic approach might be more fruitful. In the more immediate future, improved probes in the different near-infrared windows that can be used in mammalian tissue in the ~600-nm and 1,700-nm range[123] can greatly increase the imaging depth in mice already, especially if they can be combined with adaptive optics[124].

Increasing the resolution of whole-body imaging from the currently routinely achieved cellular resolution to subcellular and even molecular resolution will ultimately be possible in the absence of any fundamental physical barriers. Improvements in the working distance of high-NA optics, further advances in clearing methods and the adaptation of super-resolution methods to whole-mouse imaging will all contribute to this aim. On a more practical note, it will also be important to increase imaging speed to complete high-resolution scans in a reasonable time and to develop smart data handling infrastructures to deal with the enormous amounts of data that would be produced by such high-resolution experiments.

On the molecular profiling side, ideally, we should obtain the entire proteomic and transcriptomic information (including post-transcriptional and post-translational modifications). Currently, our ability to detect low-abundance molecules, especially in samples where a high dynamic range is required, remains limited[125]. Additional methods for increasing the detection sensitivity, especially for RNA and protein variants, will be needed to achieve the ultimate level of molecular profiling.

Finally, an important aspect is general usability, that is, nonexperts should be able to perform labeling, clearing, imaging and data analysis as easily as western blotting or confocal microscopy today. This will require the development of user-friendly clearing and labeling machines, along with easy-to-use AI algorithms. The commercialization of single-cell methods has been instrumental in propelling the transcriptomics field to its position as the major unbiased tool of biomedicine. We will need a similar push in tissue clearing, light-sheet microscopy and 3D spatial molecular profiling to enable routine large-scale data collection. Similarly, we need AI-assisted analysis pipelines that combine high precision with interpretable decision-making algorithms for common analysis tasks. Together, easily accessible experimental and computational methods will enable the routine application of end-to-end deep 3D histology in basic, translational and clinical research.

A general problem of all methods providing large-scale data is the extraction of scientific or practically actionable insights. AI, in particular, deep learning, will continue to be our major tool in translating structural and molecular data into meaningful insights that we can use to generate solutions to major problems, including treating diseases. Recent developments in organizing data, integrating and connecting different data sources and making information accessible to humans (for example, knowledge graphs, large language models, text embeddings and graph learning strategies) will need to be connected to the output of end-to-end deep 3D histology pipelines to make maximum use of the data.

Usability is also limited by the enormous computational resources required for handling, storing and analyzing the large amounts of imaging and molecular data obtained in a typical experiment and for making them available to the scientific community. We find that an integrated approach, in which the data are directly stored and analyzed on a high-performance computing cluster and then the processed data are made available to the individual researchers, works best in our hands. Still, making terabytes worth of data available to the community remains a substantial challenge in terms of computational and long-term financial resources.

In conclusion, high-resolution 3D imaging of cleared tissues will be instrumental in our quest to develop an organism-wide understanding of physiological and pathological processes. While we still face technical challenges in imaging, molecular profiling and deep learning-based data analysis, tissue clearing is already facilitating the investigation of biomedical questions while avoiding the human biases that limit our ability to discover. A continuous acceleration and automation of 3D tissue analysis from data acquisition to data analysis will therefore allow us to obtain a century's worth of insights into disease mechanisms and drug targets in just a few years.

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Competing interests

A.E. is a cofounder of Deep Piction.

Additional information

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