Morphomics via Next-generation Electron Microscopy

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Abstract

The living body is composed of innumerable fine and complex structures and although these structures have been studied in the past, a vast amount of information pertaining to them still remains unknown. When attempting to observe these ultra-structures, the use of electron microscopy (EM) has become indispensable. However, conventional EM settings are limited to a narrow tissue area that can bias observations. Recently, new trends in EM research have emerged that provide coverage of far broader, nano-scale fields of view for two-dimensional wide areas and three-dimensional large volumes. Together with cutting-edge bioimage informatics conducted via deep learning, such techniques have accelerated the quantification of complex morphological images. Moreover, these advances have led to the comprehensive acquisition and quantification of cellular morphology, which is now treated as a new omics science termed ‘morphomics’. Moreover, by incorporating these new methodologies, the field of traditional pathology is expected to advance, potentially with the identification of previously unknown structures, quantification of rare events, reclassification of diseases and automatic diagnosis of diseases. In this review, we discuss these technological and analytical advances, which have arisen from the need to analyse nano-scale bioimages, in detail, as well as focusing on state-of-art image analysis involving deep learning.
1. Introduction

It is said that ‘a picture is worth a thousand words’; in line with this sentiment, scientists have been developing tools and techniques to visualise biological specimens for around 400 years. Since Robert Hooke first published ‘Micrographia’ with beautiful illustrations of cells and living organisms in 1665 [1], the use of light microscopy has led to many important discoveries, not only of various microorganisms, including Mycobacterium tuberculosis [2] and Treponema pallidum [3], but also of cellular components, such as red blood cells [4], capillary vessels [5, 6], brain neurons [7–9] and intracellular structures including nuclei [10].

In 1932, electron microscopy (EM) invented by Von M. Knoll and Ernst Ruska [11, 12] advanced imaging in the field of biology, with one example being the microscopic observation of Escherichia coli at a magnification of 10,000 times [13]. In the 1940s, EM enabled the discovery of virus and phage particles [14, 15], which stimulated the later development of virology. Initially, the application of EM in biological research was difficult because of the lack of histological techniques [16]; however, with the development of fixing and staining methods using aldehydes and heavy metals [17–30], EM was applied more broadly to histology [31]. The use of EM has also revealed a variety of cellular functions such as autophagy [32], slit structures in kidney glomeruli [33] and undifferentiated cell states in induced pluripotent stem cells [34, 35]. Thus, the technological developments in EM revealed a new world of intracellular nano-metre-scale histology that brought with it major biological insights.

In the last decade, two important trends in EM research have primarily emerged: (1) coverage of a two-dimensional (2D) wide-range field for simultaneous capture of many cells and/or whole tissues at high resolution [36, 37]; (2) three-dimensional (3D) resolution, which provides a volumetric viewpoint and reveals the stereoscopic morphology of whole cells and the intercellular connections in tissues [37–39]. These developments potentially facilitate the handling of large bioimaging datasets and/or the collection of comprehensive morphological data from biological specimens [40–42]. Thus, EM has gained attention as a potential new omics modality. In this review, we discuss the application of ‘big data’ analysis to nano-scale bioimages and we highlight the use of deep learning for state-of-art image analysis.

2. Implications of EM Observations in Biology

2.1. EM Observations of Biological Microstructure

EM produces outstanding images of membranous cellular structures that maintain cellular morphology and contribute to intracellular/extracellular functions including intracellular transport, phagocytosis and migration [43, 44]. The cell forms a thin and flexible lipid bilayer boundary that holds both hydrophilic and lipophilic solutes, such as DNA/RNA, proteins, glycogen, lipid droplets and minerals, inside the cell [45]. Lipid membranes are also located in cellular organelles such as the nucleus, Golgi apparatus, mitochondria and endoplasmic reticulum [45, 46]. EM can capture the membrane structures, and the cytologic images show the localisation and distribution of cellular components and/or their cellular dynamics.

In practice, the structure and localisation of cellular components and organelles can be captured at the nano-scale using chemically fixed biosamples and a resin-embedded ultra-thin-sectioning EM method [47, 48] as follows. To preserve the characteristic microstructures of cell membranes and cellular solutes, specimens must undergo double fixation using a glutaraldehyde agent and osmium tetroxide [49, 50]. Osmium staining is used to selectively visualise intracellular structures as the chemical covalently binds to osmiophilic materials, such as unsaturated fatty acids and biomolecules with unsaturated bonds [29, 51, 52], thereby conferring some electron density to the osmiophilic substrates. Additional staining using heavy metals, such as lead [19, 25], acetic uranyl [23, 53], gadolinium [54] and neodymium [55], is also performed to improve the contrast of intracellular components. For the preparation of bulk
samples larger than a millimetre in size, an en-bloc staining method with improved penetration of osmium acid [52, 56–59] has been developed in addition to a prolonged staining technique used for large heterogeneous sample preparation, including the sequential osmification and treatment of samples with potassium ferrocyanide [60]. Through metal staining, cell morphology can be observed with a clear membrane contrast and the retention of cellular components.

2.2. Ultramicrotome and Resin-embedding Ultra-thin Sectioning Methods for EM

Resin-embedding before sectioning [61] is important to EM. Epoxy resin is widely used for the resin-embedding of dehydrated samples [62–64]. It can permeate into the microstructures of cells and/or tissues [65], and then the resin-infiltrated sample can be polymerised (at ~60–70°C) into a firm plastic that is suitable for ultra-thin sectioning.

EM observations of sections were first developed in 1939 [66]; however, the cutting method was difficult to demonstrate because microtome equipment, knives and embedding agents did not exist at that time. In 1948, Peace and Baker used a modified microtome to produce ~500-nm-thick sections for optical microscopy [67]. Later, Sjostrand developed a microtome with a thermal expansion feed system; it used a heater in the cutting feed mechanism and the expansion of the spindle via heating resulted in 20-nm-thick sections [68]. In addition, Porter and Blum developed a mechanical feed microtome with a minimum feed resolution of ~25 nm [69]; the thin sections they produced were the precursors to those produced using current ultramicrotomes.

Fernández-Moránab subsequently proposed a method for producing ultra-thin resin sections using a diamond knife [70–72]: trimmed resin-embedded samples were mounted on an ultramicrotome and sectioned at room temperature, usually to <100 nm, using a diamond knife with a wedge angle of 35–45° [73, 74]. Currently, commercially available diamond knives for ultramicrotomes are around 2–4 mm in width. It is challenging to grind diamond knives wider than 4 mm using a rake angle ≥45° because the cutting edge must be configured with an acute angle. In addition, the wider blade can easily spill during cutting, leading to traces and phenomena on the cutting surface such as knife marks, striations, chatter, vibration and compression [75], which become artefacts during EM observations.

2.3. Cutting Theory for Section Preparation

To maximise efficiency and success during ultramicrotome sectioning, it is necessary to accumulate cutting data as well as considering basic theory of cutting. For example, complex correlations among parameters, such as section thickness, knife angle, clearance angle, rake angle, sectioning speed and Vickers hardness, during cutting determine the quality of ultra-thin sections [76]. To improve machinability, it is necessary to reduce sectioning forces in the XYZ axis direction; this requires quantification of the resistance force during shear processing of the sample, optimisation of cutting conditions and clarification of the hardness of the sample [76].

2.4. Automatic Collection of Serial Sections

With the advent of the 3D EM methods, the preparation of serial sections from specimen blocks has become possible. Generally, serial sections are prepared using a conventional ultramicrotome by floating the sections on the water surface using a boat attached to a diamond knife [77]. When several sections have accumulated, they are manually scooped out of the water using a glass or silicon substrate. However, it is currently difficult for even expert technicians to manually prepare and collect several hundred serial sections without missing pieces, and the process has low reproducibility. Furthermore, collecting a large number of sections is time consuming [78] and the position of the collected sections varies. Consequently, new serial-sectioning methods have been proposed. One such method involves the use of an automated...
tape-collecting ultramicrotome (ATUM), a device that automatically collects serial tissue sections using an ultramicrotome and magnetic polyimide tape [79, 80]. The ATUM devices are mainly used in connectome research, which aims to elucidate the network of neurons in the entire brain [81, 82], but they may be used in other research fields. Recently, automated ultramicrotome techniques and diamond knives particularly for use in continuous section preparation have been developed [83]; thus, several hundred to one thousand serial sections can be prepared and collected. Further details are outlined in Section 5.

Current ultramicrotomes are limited in terms the width of sections that can be cut (1–2 mm) and the range of movement of the z-axis of the device. Challenges also exist with high-throughput section preparation and multi-specimen processing. To resolve such problems, the Yagishita Giken Co., Ltd. (Wako, Japan) and RIKEN research groups are developing an ultra-thin section preparation system that can prepare and collect large-area ultra-thin sections by combining machine elements and machining tools for precision cutting. For example, a microtome equipped with a new cutting technique is being developed; in this ultramicrotome system, the aim is to control the cutting feed axis and infeed axis with high repeatability and positioning performance. The recovery system for ultra-thin sections should be able to numerically control the running speed to match the stable tape running speed and the intermittent generation speed of the sections. A synthetic diamond knife has also been developed with a blade width of 10 mm, a blade angle of 50° and stability in terms of material and quality. To obtain stable ultra-thin sections, it is necessary to optimise the processing conditions by observing the cutting surface properties of the specimen and measuring the cutting resistance force. In time, additional researchers from various fields are expected to contribute to morphology research and accelerate the development of technology in this field.

3. Transmission EM for Wide-area Imaging in Biology

3.1. Conventional Transmission EM and Its Challenges

In biological and medical fields, EM observations typically involve bioimaging of stained thin sections of plastic-embedded samples using transmission electron microscopy (TEM) [84, 85]. In TEM, the device accelerates an electron beam with an extremely short wavelength and irradiates the thin section. Through detection of the transmitted and forward-scattered electrons through the thin section [86], a 2D projected magnified image of the specimen can be obtained at a sub-nano-scale resolution (Fig. 1a). Specimens such as bulk tissues must be sufficiently thin to allow electrons to pass through. The ultra-thin sections are typically ≤100-nm-thin because thicker sections cause inelastic electron scattering [87]. Such sections are placed on a metal mesh grid for observation [88].

Some limitations exist when attempting to prepare ultra-thin slices suitable for TEM observation. First, the metal mesh itself interferes with observations of overlapping tissues [88, 89]. Second, the brittle slices are prone to breakage, which restricts imaging time. Third, since large specimens do not fit in a single field of vision (FOV) of the microscope [85, 90], a controlled system is required to automate imaging process [91] and a handling system is needed for large-sized digital images [42]. These limitations have restricted the use of conventional TEM when observing narrow areas or a relatively small set of cells [84]. Consequently, the current application of EM in clinical diagnosis is limited to assisting diagnosis of renal diseases, undifferentiated tumours, metabolic diseases that mainly affect the muscles or nerves and diseases with unknown aetiologies [85, 92].

Some progress has been made in overcoming the limitations associated with TEM observations. First, a large-sized window and tough supporting film with uniform thickness have been developed to assist with observing wide-range areas [37, 93]. One such supporting film, the LUXfilm® support film, is a highly transmissive and robust film that is better suited for automatic TEM workflows; however, it produces substantial noise without any noise
reduction [94]. Konyuba et al. proposed a large-scale silicon nitride window chip deposited using low-pressure chemical vapour deposition as a new support grid for wide-area TEM imaging [95]. This chip is mesh-free, which allows wide-area support for the specimen without creating imaging interference.

A large number of digital TEM images can be captured using an auto-acquisition system with the device [96, 97]. When the physical movement on the microscope stage is not sufficiently precise to obtain the required imaging resolution [98], computational registration and stitching techniques of digital images can be used; these reconstruct single-captured wide-area images from individual tile images [90, 99–107]. These tiled images are also known as montage or mosaic images. Toyooka et al. reported the use of wide-area TEM imaging with a tiled scan of a whole plant cell; this technique successfully produced 3,000–5,000 digital images with the desired range of observation and comprehensive detection of plant organelles [91, 108, 109]. Bock et al. reported electron micrographs of an entire 120,000 × 80,000 pixel thin section of the mouse visual cortex using controlled automated x–y stage motion and image acquisition [37]. Faas et al. performed large-scale EM analysis known as virtual nanoscopy, a methodology for ultra-structurally mapping regions of cells and tissues as large as 1 mm2 at a nano-metre resolution [36]. Lamers et al. imaged human severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-infected intestinal organoids autonomously using virtual nanoscopy slides and TEM tomography [110]. As shown in Fig. 2a, through wide-range TEM imaging, including TEM [JEM-1400/Matataki Flash Camera (2,048 × 2,048 pixels), Jeol Ltd., Japan] with a silicon nitride window chip and an automated montage system on Limitless Panorama software (Jeol Ltd., Japan), it was possible to obtain a view of mouse glomeruli that consists of 8,500 tiled images. Using this technique not only preserves the conventional resolution required to capture the basement membrane of the glomeruli and podocyte foot effacement but also enables imaging of multiple glomeruli within the same captured view.

3.2. Scanning-type TEM for Large-scale Microstructural Imaging

The nanotomy project (http://www.nanotomy.org/) and its related works provide systematic virtual nanoscopy studies mainly using scanning-type TEM in which the electron probe is scanned across the sample and the transmitted signals are detected point-by-point to form an image [111]. Accordingly, large-scale morphological views of various biosamples, including human pancreas tissue with type 1 diabetes [112], human autoimmune blistering disease pemphigus [113], human skeletal muscle biopsies with histological minimal myositis and capillary pathology [114], SARS-CoV-2 in human tissues [115], human haematopoietic stem/progenitor cells [116], zebrafish brain tissue [117], a free-living marine flatworm [118], rat islets of Langerhans [88, 119–121], mouse salivary gland organoids [122], mouse skeletal muscle [123], mouse kidneys and glomeruli [124, 125] and hamster liver sinusoids [126], have been demonstrated. Recently, Dittmayer et al. developed a methodology for preparing large-scale digitised samples designed to acquire entire sections free from obscuring flaws using scanning EM in the transmission mode; this technique will substantially improve information and throughput gain when analysing experimental and/or clinical samples, including diagnostic muscle, nerve, and kidney samples [127].

Large-scale EM with energy-dispersive X-ray (EDX) analysis enables the acquisition of elemental composition patterns from the surface of samples as well as the visualisation of traditional grey-scale EM images for composition-based interpretation [128, 129]. EDX analysis of the rat pancreas has been used to distinguish, for example, cytoplasmic mitochondria and granules via elemental fingerprinting [120]; thus, analysis of disease tissues may also be possible using composition-based EM. In the objective identification of human pancreas cell types with type 1 diabetes, element maps of granule content produced using EDX analysis have provided data on the elemental variation of granule content within each of the aforementioned
cell types [112]. Thus, EDX analysis enables unbiased fingerprinting of cell types and the functionalities of each cell can be inferred from elemental fingerprinting.

4. Large-scale Bioimaging Using Scanning EM
4.1. Application of Scanning EM in Biology and Recent Progress

Scanning EM (SEM) involves the use of a different type of electron microscope to that used for TEM (Fig. 1b). According to reports, SEM was developed in 1965 [130, 131], about 30 years after TEM. In SEM, the incident electron probe scans across the surface of a specimen in a raster fashion [132], and the interaction between the relatively heavy elements containing the sample and the impacted electrons produces various types of emissions including secondary electrons, backscattered electrons and characteristic X-rays [132]. By detecting such emission types, SEM creates images that reflect the topological contrast or compositional information of specimens as signal intensities in the digital images [132, 133]. Because typical SEM measurements do not require the transmission of electrons through the sample, the process can be used for surface observation of semi-thin sections [134, 135] and bulk specimens [136] such as the surface of kidney glomeruli [137–142], microbial adhesion [143, 144], cell adhesion [145], virus-infected cell surface [146] and blood cells [147–151]. However, traditional SEM images lack the characterisation of internal ultra-structures due to the relatively lower signal under high-magnification imaging conditions [134, 136, 138, 140, 150]. Thus, the application of SEM in diagnostic pathology is limited [152].

Fortunately, recent progress in SEM imaging [153] has led to new SEM utilities, e.g. scanning across ultra-thin sections of resin-embedded specimens under conductive support [154–156]. Scanning electron microscopes equipped with a cold-field emitter [153, 157], a Schottky emitter [158, 159] or a thermal-field emitter [160] as an electron source are known as field-emission scanning electron microscopes; they produce high-resolution images because of the smaller spot size from these emitters [132], the negative stage bias potential [161] and the improved sensitivity of the multiple electron detector system [132, 162, 163], even when the ultra-thin tissue sections are <100 nm. The backscattered electron detection by SEM when using resin-embedded ultra-thin sections provides a reverse contrast of the view that is conventionally possible when using TEM [164, 165]. Although the contrast of the backscattered images is reversed, the quality of the images is sufficient to enable general morphological analysis from TEM observations [85, 166–173]. Furthermore, SEM observations provide high-resolution histological images that are independent of section thickness through regulation of acceleration voltage. Observation of tissue sections through a combination of SEM and resin-embedded sectioning is also known as the section SEM method. In addition, other SEM methods, such as helium ion scanning microscopy [174–177], transmission-mode SEM [178, 179], atmospheric SEM [180] and environmental SEM [181, 182], have been developed.

Recently, the popularity of SEM has lowered the accessibility of the usage and potentially lowered the operating costs [183]. For surface observation with the SEM, various shapes of sample stands can be used provided that they are not made of electrically charged materials. A silicon wafer is a typical base used for biological SEM specimen observation [184]; indeed, huge specimen bases, e.g. 10-cm-diameter wafers, are available. Silicon wafers also adhere well to ultra-thin sections. Sections scooped up on the wafer can be stably stored even when the section is large [50]. In addition, glass slides that are inherently prone to static electricity can be used under conductive treatment by applying a metal coating to the slides before SEM observations are performed [185, 186]. Because SEM has typically been used for surface observation of bulk samples, the sample storage space is designed to have a large XYZ dynamic range. Such advantages of the conventional technique can be fully exploited with the improvement of SEM devices. In other words, the stable fixation of the sample on a sample board has made it possible to observe samples over a long period. The large XY dynamic range
facilitates the introduction of relatively large sections (i.e. several millimetres), multiple sample sets and hundreds of sections into the instrument at the same time. The use of the range in the z-direction has made it possible to include microfabrication methods, such as knife cutting and laser cutting, in the sample chamber of the scanning electron microscope [164, 187, 188].

4.2. Wide-area Imaging Using SEM

With the aim of practically producing a fish-eye perspective view, early panoramic imaging with SEM was performed in the 1990s [189]. To convert the mosaic images of SEM into a combined image, montage capturing software and image stitching algorithms were developed, similar to existing software for microscopy images [102, 103, 190–195]. For wide-range SEM imaging, Brantner et al. demonstrated large-area and high-resolution mosaic imaging of a 2.5 × 1.8-mm mouse spinal cord resin section (a biologically relevant scale) using the workflow of Chipscanners’s laser interferometer stage, FOV mapping and an image stitching technique [196]. Kataoka et al. indicated that stitching SEM enabled the observation of an entire pulmonary alveolus with influenza virus particles in a resin section [197]. More et al. applied a montage SEM imaging technique to quantify the number, myelination and size of axons in the rat fascicle using a computer-assisted axon identification and analysis method [198]. Maeda et al. reported the results of cell counting with autophagy-like vacuoles in wide tissue fields (~600 images in a total area of 0.25 mm2) of the mouse cortex using an automatic acquisition system for tiling SEM images [50]. Kume et al. reported an imaging database of wide-range montage SEM images and their metadata for various tissues, including those from the kidneys, liver and brain cortex region of rodents and human cultured cells [42]. Figure 2b shows imaging data obtained using wide-area montage SEM images of a rat liver. We integrated more than 1110 images to reconstruct the rat liver leaflet in this large-area image (1 x 0.6 mm). Strikingly, we were able to observe the whole liver lobule while preserving the spatial resolution in EM. The image information obtained using wide-area EM is substantial, which makes interactive visualisation difficult. To solve this problem, image data is converted to the Deep Zoom Image format, which is a layered format on a pyramid structure; this allows interactive zooming in and out for improved visualisation using web software such as Google street view and OpenSeadragon [36, 42, 100]. The use of wide-area EM imaging avoids arbitrary selection of target regions in experimental or diagnostic specimens, and it enables the efficient and comprehensive observation of biological tissues in a time-efficient manner without susceptible bias.

4.3. Multibeam SEM

SEM imaging is sometimes more time consuming than TEM imaging due to raster fashion scanning. Thus, methods for speeding up SEM imaging have been developed as follows: (1) image capture with a higher speed single beam, (2) imaging different sections in parallel on multiple EM devices and (3) parallelised imaging of the same section using multiple scanning beams. As a method of parallelised imaging, Eberle et al. demonstrated a throughput imaging technique with multibeam SEM [199–201]. In this system, 61 electron beams are scanned over the sample with one global scanner and secondary electron signals are acquired for each scan position of each beam [199–201]. The multibeam SEM then produces 61 montaged SEM images simultaneously as a hexagonal FOV. In the resultant images, all membranes of neural tissue were clearly visible and intracellular organelles were distinguishable [199]. One hexagonal FOV is used to image a region of about 100 μm2; however, by performing montage imaging, a region ≥1 mm2 can be imaged [199]. Pereira et al. reported that a surface area of 5.7 mm2 could be imaged in a human femoral neck tissue sample, resulting in 897 hexagonally shaped multibeam FOVs comprising ~55,000 high-resolution image tiles and 75,000 megapixels [100]. Multibeam SEM with 196 electron beams has also
been developed; this SEM device was designed to detect transmission electrons and backscattered electrons [202]. Thus, multibeam EM systems contribute to high-speed collection of digital images. The applications of multibeam SEM include a much wider-range 2D imaging in addition to 3D EM analysis and brain connectomics research [81, 82, 203, 204].

5. 3D Resolution Bioimaging using EM

5.1. Implications of 3D Resolution for Ultra-microstructural Observations

To obtain histological and cellular images of targeted 2D regions, the use of ultra-thin section EM techniques with resin-embedded samples is widely accepted and has led to new biomedical discoveries [31]. Indeed, a cellular image obtained from only one tissue section contains substantial biological and medical information. The steric and complex communication of many cells allows living tissues to exhibit and maintain their function [81, 205, 206]. Occasionally, the appearance of characteristic cells and compositions in diseases serves as a biomarker for disease identification [112, 207]. However, the thickness of ultra-thin sections is 50–200 nm; assuming that the actual size of a cell is ~10 µm, one ultra-thin section can be used to interpret cellular events in around one-fiftieth to one-two hundredth of the total cell volume. In most cases, even within the same cell, the shape of the cell nucleus differs greatly depending on the cutting angle and position of the cross section (Fig. 3a). In other words, when a cell image is observed in a cross section, it is difficult to precisely describe whole-cell morphology. In addition, there is an ongoing debate among researchers as to whether the cellular view obtained from extremely thin sections contains artificial cutting bias such as compression. In such cases, visualising the entire morphology of target cells or tissue regions in 3D resolution is required. To realise 3D-directed resolution in EM techniques, observing multiple tomographic images for each cross section one-by-one is a reasonable method [208, 209]. Thus, it is expected that the generalisation of stereoscopic EM techniques will lead to a deeper understanding that would otherwise not be obtained using conventional 2D EM techniques. Here, we reviewed the morphomics techniques used to obtain volumetric EM images (Fig. 4).

5.2. Focused ion beam SEM

Focused ion beam SEM (FIB-SEM) is used to observe the surface of a specimen milled by an ion beam on the sample stage of the scanning electron microscope [210]. By repeatedly and alternatively exposing and imaging the new top surface, serial images are captured, although the cutting surfaces cannot be preserved (Fig. 4a). FIB-SEM offers the best z-axis resolution at 4–5 nm; thus, it is suitable for mesoscale observations such as for the observation of cellular organelles [211, 212]. FIB-SEM was originally applied in the 1990s [213], at which time the area covered by ion beams was far smaller than it is today. As the area of observation is enlarged in FIB-SEM, it is commonly applied to various biological samples. Moreover, the outstanding z-axis resolution of FIB-SEM has seen it applied for observations of intracellular events and organelles [214] including in yeast [215], the rodent brain [164, 212, 216], mouse scleral fibroblasts [217], mouse bone marrow adipocytes [218], mouse periodontal ligaments [219, 220], rat kidney glomerular endothelium [221], rat glomerular podocytes [206, 222], human skin fibroblasts [223], human lung epithelium [224], glandular epithelial cells [210], mammary gland organoids [225] and primary mouse pancreas β cells [226]. Using FIB-SEM, Miyazono et al. demonstrated dramatic mitochondrial structural changes that were triggered by the loss of mitochondrial membrane potential [227]. Notably, Xu et al. enhanced the FIB-SEM system by accelerating image acquisition; the speeded-up system allowed imaging of a Drosophila brain at 106 µm³ [228, 229], which serves as a powerful dataset in brain connectomes. Furthermore, Xu et al. reported volumetric image datasets of whole cellular architecture with the finest
possible isotropic resolution (about 4 nm square voxel) using FIB-SEM [230], provided as open access data via OpenOrganelle (https://openorganelle.janelia.org/) [214, 230], which allows the study of comprehensive cell morphology [226, 231].

5.3. Serial Block-face SEM

Serial block-face SEM (SBF-SEM) is used to observe an exposed sample surface cut using a built-in diamond knife [232]. Compared with FIB-SEM, SBF-SEM facilitates the handling of a broader area as well as faster sample sectioning. SBF-SEM produces 1,000 3D EM images, but it cannot preserve processed sections (as with FIB-SEM) (Fig. 4b).

The prototype of SBF-SEM was produced in 2004 by Denk et al. [233]. They not only showed the power of the technique to reconstruct 3D tissue nano-structures but also directed visualisation of neural circuit reconstructions in neuroscience research [164, 234]. In brain research, the largest mammalian cerebral cortex dataset yielded a reconstruction ~300-fold larger than that in previous reports, which allowed the analysis of axonal patterns [235]. In kidney analysis, Ichimura et al. revealed novel 3D structures in rat podocytes [206, 236]. Other reports also have shown the feasibility of SBF-SEM in 3D EM studies including studies of Drosophila epithelium [237], zebrafish blood vessels [238, 239], mouse B cells [239, 240], rat livers [205] and mouse and human kidneys [241–244]. SBF-SEM has also been applied to image the whole structure of yeast [245, 246], Trypanosoma [247] and cultured cells [248–251].

5.4. Array Tomography

Array tomography (AT) is also used to achieve stereoscopic EM (Fig. 4c). In the AT method, serial ultra-thin sections are prepared from a resin-embedded block using an ultramicrotome and then the same site for each section is observed sequentially using TEM or (primarily) SEM [252]. A continuous tomographic image is then reconstructed to obtain the 3D tomography. Unlike other methods, the AT method is notable for its capacity to preserve thin sections, which could then be re-observed later. The resolution of the z-axis in the AT method is the thickness of the section, which is approximately 50–100 nm. Combining the AT method with SEM potentially allows for wide-area volumetric observations [40]. This technique is also known as the serial-section SEM method [184].

The idea for creating serial sections dates back to the 1950s, soon after the first ultramicrotome became available [77]. Ribbon-like serial sections were transferred to supporting grids for observation. Later, in a 1970s report on 3D construction of the juxtaglomerular apparatus of the rat kidney, 500 serial sections were obtained and TEM observations were successful [208]; however, 3D illustrations were limited. Subsequently, TEM was used for 3D EM, especially until the 2000s. Serial-section TEM (ssTEM) images have been acquired from serial sections of differentiating monocytes [78], neuron connections [253], yeast cells [254] and the endoplasmic reticulum [255]. Notable results of AT and TEM combined include whole-imaging of an adult Drosophila brain using a custom high-throughput ssTEM platform developed by Zheng et al. [256]. This volumetric morphology obtained by ssTEM has contributed to mapping brain-spanning circuits and accelerated research in the field of neuroscience.

In parallel with the development of ssTEM, AT combined with SEM was proposed in 2007 [257]. This combination method has been used to study varicella-zoster virus-infected cells [258], the Golgi apparatus in different cell types [259] and sorted immune cells [260]. In addition, we could successfully generate a 3D volume EM image of a human leukaemia cell and the macula densa in the distal tubules of a mouse kidney glomerulus using AT combined with SEM (Fig 3bc). The SEM-based serial-sectioning method is suitable for relatively large samples because it collects larger-area serial thin sections onto the silicon substrate or glass slide [184, 261]. However, the AT method is generally challenging because it is difficult to
manually prepare continuous ultra-thin sections of hundreds to thousands of samples. To improve the AT technique, customised AT methods have been developed such as magnetic collection of ultra-thin sections [203], customised substrate holders [40, 262, 263], a modified AT-boat diamond knife [83], tape collections of sections [79, 81, 264, 265], a carbon nano-tube tape for serial sections [82] and the combination of semi-thin serial sectioning and FIB-SEM [266]. Among these, the tape collection system using ATUM has facilitated automatic collection of tissue serial sections and volumetric SEM [79, 267]. This ATUM-based AT–SEM method was used to clarify the sub-volume of the mouse neocortex from ~2,000 serial sections [81] as well as all myelinate axons of the zebrafish brain from 16,000 serial sections [268]. Morgan et al. imaged 10,000 sections of the mouse visual thalamus, which were produced using ATUM to a thickness of 30 nm, with an imaging volume for the dataset of 0.8 mm × 0.8 mm × 300 µm; 899 synaptic inputs and 623 outputs were mapped in one inhibitory interneuron [269]. Witvliet et al. used the ATUM–SEM system to reconstruct the full brain of eight isogenic Caenorhabditis elegans individuals across postnatal stages in an age-dependent manner [270], which provided insights into the mechanism of connectome development during brain maturation. These obtained datasets are also provided in BossDB (Brain Observatory Storage Service & Database, https://bossdb.org/) [271–273]. Recently, Shapson-Coe et al. applied a combination of ATUM-based AT and multibeam SEM to petabyte-scale large volume imaging of the temporal lobe of the human cerebral cortex, and they computationally rendered the 3D structure of 50,000 cells, hundreds of millions of neurites and 130 million synaptic connections in the volumetric images; their findings suggested the existence of a new subset classification of deep-layer excitatory cell types [274]. Moreover, the use of such large-scale stereoscopic EM techniques to analyse the microstructures of pathological conditions is expected to improve our understanding of disease-specific structures that could not be obtained using conventional EM techniques.

5.5. 3D Imaging using High-Speed TEM

To develop high-throughput TEM imaging, Graham et al. used a tape-based, reel-to-reel pipeline that combines automated serial sectioning and a TEM-compatible tape substrate, GridTape [275]. This acquisition platform provides nano-metre-resolution imaging at fast rates via TEM. Based on this pipeline, multiple-scope parallel imaging using a 50-MP camera has enabled image acquisition of a >1-mm³ volume of the mouse neocortex, spanning four different visual areas at synaptic resolution, in less than 6 months; in turn, this has yielded a >2-petabyte dataset from over 26,500 ultra-thin tissue sections [265]. In addition, Phelps et al. applied GridTape-based serial-section TEM imaging to acquire a synapse-resolution dataset containing an adult female Drosophila ventral nerve cord [264]. The complete connectivity maps provided a deeper understanding of how the nervous system controls the locomotor rhythms underlying swimming and crawling [264]. Since TEM offers much faster imaging compared with that of SEM, this research could be applied in areas that require broad observation with precise imaging.

6. Correlative EM and Multimodal Imaging

6.1. Correlative Light Microscopy and EM

The body can be understood more deeply if tissue functions and macromolecular fingerprinting can be estimated at the nano-level, which is sometimes difficult to achieve using only EM-based morphomics. For example, distinguishing between excitatory and inhibitory neurons cannot be achieved based only on morphology [276, 277]. To resolve this issue, correlative EM, which combines EM and other imaging tools, can be used to better understand molecular functions and other factors. Correlative EM is also useful for screening or targeting specific structures, especially when targeting cellular markers. A well-established example is
the combination of light microscopy and EM, known as correlative light and electron microscopy (CLEM) [278]. The idea behind CLEM was first proposed in the 1980s [213]. Samples are initially imaged using a light microscope to detect histological morphologies or fluorescence signals, after which they are subjected to EM imaging with a nano-resolution. Correlation imaging is achieved either by sharing the same FOV for both modalities or by sample transfer in tandem [278].

To screen for structures of interest, Ronchi et al. developed a workflow that combined fluorescent labelling and FIB-SEM, which enabled correlative targeted imaging of animal mammary gland organoids, tracheal terminal cells and ovarian follicular cells [225]. CLEM has also been applied to the mouse brain [252, 279–283] and the ferret brain [284] as well as whole model organisms [83], cultured cells [227] and various tissues [232]. In addition, CLEM could be used for in vivo multicolour imaging, known as Brainbow [285–287].

When targeting specific structures, Trzaskoma et al. applied CLEM to reveal 3D chromatin folding [288]; they combined DNA fluorescent in situ hybridisation with SBF-SEM. Oorschot et al. published a workflow integrating the Tokuyasu technique to preserve the antigenicity of proteins and investigated neural stem and progenitor cell populations [276]. In addition, 3D CLEM combined with the CryoChem technique allows for quality ultra-structural preservation that is broadly applicable to cultured cells and tissue samples [289]. The CLEM method can even be used to target specific proteins under transgenic conditions via the engineered peroxidase gene APEX2 [290–293], which serves as a labelling probe in both EM and light microscopy [294]. This APEX2 system has been successfully implemented to track lysosomes in dendrites [295] and to visualise the localisation of endoplasmic reticulum chaperonin [296], the outer endoplasmic reticulum and mitochondrial membrane [297], membrane proteins [298] and multicolour labelling of peroxidases [299]. Recently, the APEX-Gold method, which has high sensitivity, was used as a genetic tagging in 3D EM [300].

For correlation of live cell imaging, Fermie et al. analysed the dynamics of individual GFP-positive structures in HeLa cells and then correlated these with images from FIB-SEM [301]. Thus, they overcame the limitations of EM, i.e. that EM cannot visualise live cells. Betzig et al. first introduced a combination of super-resolution light microscopy and EM (super-resolution CLEM) to image specific target proteins in the thin sections of lysosomes and mitochondria [302]. Currently, super-resolution CLEM can achieve a resolution of 20–50 nm, although the distortion of the sample becomes a problem at <10-nm resolutions [303]. This technique was also utilised to visualise the Golgi apparatus [304, 305], mitochondria [306, 307] and other organelles [306].

### 6.2. Correlative X-ray Computed Tomography and EM

X-ray computed tomography (CT) has been applied to biological tissues or cells to obtain almost single cell-level morphology data [308]. In practical terms, observing cells that are approximately 10 µm in size requires sub-micro-resolution potential in the CT device. When observing intracellular structures, the use of synchrotron radiation X-ray is necessary. Although, at present, single-cell imaging with a CT device remains a special case, in this section we discuss cellular tissue analysis, including single-cell imaging, using CT devices and further correlative CT-EM.

The following are a few examples of X-ray CT applied in biology research to date. In the early 1980s, the micro-CT technique was developed to achieve 3D observations at micrometre resolutions [309]. This technique can be used to obtain a projection image of a sample by irradiating it with X-rays with wavelengths of ~1 pm to 10 nm. Compared with the 1–2 mm resolution in conventional medical CT scans, micro-CT tomography results in a higher spatial resolution of 1–50 µm (generally approximately 5 µm resolution per voxel) [310, 311]. Because the spatial resolution depends on the focal spot size of the X-ray source [312, 313],
relatively small sample pieces (<10 mm in size) can be used for micrometre-level resolution with micro-CT. Moreover, micro-CT imaging provides high contrast results, especially in tissues with high or low X-ray permeability (e.g. the lungs or bones, respectively), without the need for special sample preparation. However, particularly in soft tissues, including the brain and renal cortex, suitable staining techniques are required to increase absorption-based contrast of tissue structures [314–316]. In several studies, micro-CT has been applied to visualise juvenile coral [317], small organisms [318–321], nano-material in lung tissues [322], mammalian brain tissue [310, 323], rodents kidney nephrons [316, 324, 325], mouse liver structures [326], mouse embryos [327–332] and human placenta [333]. In addition, the phase-contrast approach of X-ray CT can be applied generally to unstained specimens such as mouse kidneys [334], the human heart [335], human brain tissue (cerebellum) [336] and plant germination [337]. Overall, this modality has become a promising method used in morphomics.

For 3D non-destructive targeting of a region of interest in a specimen, EM analysis correlated with the X-ray CT modality has been proposed. Several reports of correlative CT and other microscopic techniques have included EM [338–341]. In particular, correlative micro-CT and EM has been applied to clarify neural 3D structures in mouse brain tissues [342]. Silver impregnation staining applied to neurons can also feasibly be used in correlative workflows [343, 344]. Karreman et al. demonstrated the in vivo tracking of single tumour cells using multimodal imaging including X-ray CT and EM [345], which is expected to have broad applications in various biological fields.

Some CT devices even offer sub-micro-resolution [346], i.e. nano-CT, which is sometimes used as a synchrotron radiation-based CT setting and a use of soft X-ray with relatively low penetrating power. Nano-CT has been applied to reconstruct the neural network in Drosophila or the rodent brain [347–349]. In addition, this resolution can achieve cell-level observations [316, 326] that could further facilitate correlative analysis in combination with EM. Interestingly, Kuan et al. demonstrated that X-ray holographic nano-tomography can be used to image large-scale volumes with sub-100-nm resolution in Drosophila melanogaster and mouse nervous tissue, thereby enabling a close reproduction of the EM images [350]. Moreover, multiple scanning technique can comprehensively catalogue mechanosensory neurons and trace individual motor axons from muscles to the central nervous system [350]. Nano-scale X-ray CT can then bridge a key gap that helps move toward EM resolutions. Furthermore, the integration of nano-scale CT and EM has been used to study mitochondrial morphology in relation to drug resistance in human colon carcinoma cells [351]. A parallel-beams CT method can achieve much faster image acquisition and comparable or even better resolution [352]. In addition, synchrotron-based CT has been used for cellular-level analysis of bacteria [353], yeast [354, 355], mammalian cells [355, 356], neuroanatomy [357], renal microvasculature [358] and human bones [359, 360]. Overall, the X-ray CT modality has the potential to be used not only for regional targeting prior to correlative EM analysis but also for morphomics analysis of parenchymal morphology with nano-scale resolution.

7. Large Bioimage Datasets and Comprehensive Image Analysis
7.1. The Morphome and Morphomics

The morphome or biological morphome refers to the totality of the morphological features in a species [42, 361–364]. The morphome is expressed as the sum of a species’ molecular dynamics [362, 363] including its DNA (genome), gene expression (transcriptome) and metabolic (metabolome) information such as lipids and sugars (Fig. 5). It also refers to morphological phenotypes. Most morphological data are imaging data, which at first glance differs from the genomic sequences and numerical data that are mainly used as omics data in molecular biology [364]. Thus, different approaches are required to handle morphome data.
As EM device technologies have advanced [132], the acquisition speed of EM imaging has dramatically accelerated and EM-based imaging techniques have been used to study the complexity of organisms at high-level 2D and 3D resolutions. Indeed, imaging data can be produced at a level comparable with genome data obtained via next-generation sequencing. For example, wide-area imaging produced using single-beam SEM can acquire several tens of gigabytes of data in a single day of imaging [42], whereas 2D/3D imaging produced using multibeam SEM can acquire hundreds of gigabytes or a terabyte scale of image data [201]. Moreover, the latest EM methods, such as high-speed TEM methods [265] and ATUM-based AT and multibeam SEM methods [274], can generate petabyte-scale image data.

These levels of imaging data can be used to systemically measure and quantify large morphological fingerprints and diverse biological phenomena. Further quantitative morphology analysis could be applied to study biological functions. Such comprehensive approaches have led to the treatment of the resultant large imaging datasets as new omics information, which is termed morphomics (Fig. 5). This involves the integration of comprehensive (big) morphology data and bioimaging informatics, which will result in the discovery of unknown features, but there is still a bottleneck of imaging data mining. In the following subsections, we discuss such morphome analysis, outline imaging data operations and highlight image data analysis using deep learning.

7.2. Toward the Handling of Massive Bioimage Datasets

Just over 20 years ago, film photographs were still in mainstream use as EM images [365], whereas the EM images of today are high-resolution digital images. In this period of development, advances in infrastructural technologies, such as digital image-archiving, faster network communication, improved computing performance and increased storage disk capacity, have facilitated the acquisition of large-scale digital bioimage data and enabled the practical handling and processing of images [366]. It is now possible to operate with hundreds of gigabytes or terabytes of images even in a laboratory setting. Efforts are being made in the field of bioimaging data operations to handle such huge image datasets for data repositories, data sharing and reuse in a standardised manner [367].

Open-source data and data accessibility are critical to the sharing of bioimaging data [368]; fortunately, worldwide access to data has become possible via the Internet [369, 370]. However, it is necessary to construct a descriptive format and data repository, so-called metadata and an image database, respectively, prior to distributing bioimaging data [371]. The Open Microscopy Environment (OME) consortium is working to produce imaging metadata and public image archives in the medical and life sciences [372, 373]. The OME is an open-source software framework developed to address standards for sharing image data and analysis results [368, 374]. Within its framework, OME Remote Objects, an open-source interoperability toolset for biological imaging data [375], and OME metadata [376–379] have been developed to manage multidimensional and heterogeneous imaging data mainly from light optical microscopy. However, standardised metadata that describes EM experiments, including bioresources, measurement conditions and image formats, has yet to be developed; thus, integrated analysis of imaging data with other metadata has remained difficult [380]. Therefore, we previously proposed the development of microscopy metadata to describe EM experiments and their image datasets based on the data model of OME metadata [369, 380], and we offered a combination of an ontology-based imaging metadata database and an image viewer, which were distributed in a machine-readable web form [42]. At present, metadata arrangements for bioimaging, including EM, have been discussed internationally toward the reuse of microscopy data [381]. In future work, the application to medical imaging research, such as MRI and PET/CT, for human subjects will also be important [382, 383].
In May 2021, the Global BioImaging (GBI) consortium proposed criteria for globally applicable guidelines related to the tools and resources of open image data in the fields of biological and biomedical imaging [367]. The GBI also founded international non-boundaries to develop common imaging and data standards that promote data sharing and open data, as well as world-class training programmes and repositories of image data analysis tools for use by imaging scientists. Furthermore, the ‘Quality Assessment and Reproducibility for Instruments and Images in Light Microscopy’ initiative recently proposed the establishment of guidelines for quality assessment and reproducibility related to microscopy instruments and images [384]. These activities are expected not only to improve the overall quality and reproducibility of data across the microscopic bioimaging field but also to enable handling of huge bioimage datasets in a standardised manner. In addition, the barriers to data sharing of EM images should also be reduced.

As a prototype of the first open online repository to link imaging and molecular data, Williams et al. launched the Image Data Repository (IDR; https://idr.openmicroscopy.org/) in 2017 [373]. This platform stores bioimage data from several imaging modalities, including multidimensional microscopy and digital pathology [373], while integrating imaging studies and phenotypic information. It can be searched according to the metadata or image attributes and consists of two major categories: Cell-IDR and Tissue-IDR. Currently, the IDR platform distributes some EM datasets including cultured cell chromatin [385], intestinal organoids [110], budding yeast [386] and zebrafish embryo sagittal sections [36].

As a public archive of EM images, the Electron Microscopy Data Bank [387, 388] was launched in 2002 to provide a public repository of mainly electron cryo-microscopy volume maps and tomograms including macromolecular structures, such as proteins and their ligand complexes, and subcellular structures. In 2016, the Electron Microscopy Public Image Archive (EMPIAR; https://www.ebi.ac.uk/empiar/) [389] was published. This archive became a public resource for raw images underpinning 3D cryo-EM maps and tomograms. Most EMPIAR datasets are particle images and 3D tomograms of macromolecules obtained using cryo-EM, but they currently contain several 3D EM datasets of epoxy-embed tissue and cell samples obtained using SBF-SEM or FIB-SEM. For example, a 3D imaging dataset of the HeLa cell line obtained using SBF-SEM (EMPIAR-10094) [251, 390] consists of 518 cross-sectional images with a size of 8,192 × 8,192 pixels; the dataset is nearly 130 gigabytes in size, indicating that the EMPIAR covers a wide range of biological samples. Notably, all data archived in the EMPIAR is under CC0 licence and can be re-used freely without any conditions or restrictions.

To date, the development of these public bioimage resources is at an early stage and further accumulation of imaging data and development of integrated platforms is highly desirable. Representative list of current public EM datasets is summarised in Supplementary Table 1. In the past, providing large-scale open-source genomic sequence data helped advance our understanding of genomics. Accordingly, increasing the availability of imaging datasets will further stimulate imaging research and the development of novel imaging technology.

7.3. Bioimage Analysis Using Deep Learning

Little progress has been made in the methods used to analyse EM bioimages over time; conventionally, every image was examined manually [391]. EM bioimages possess a low signal-to-noise ratio, black-and-white contrast and a variety of morphological features. When using classical image analysis methods, it has been difficult to recognise and decode EM bioimages. For example, in semantic segmentation, which is used to extract a particular region in an image, the use of classical deductive methods [392–394] has failed to identify a mathematical solution for the morphological features of a particular region among various other morphologies. In most EM research cases, comprehensive quantification, including automatic segmentation, could not be achieved even after acquiring large-scale image datasets. In an
attempt to resolve these issues, current best practice is to apply cutting-edge informatics or artificial intelligence (AI) approaches [395, 396] to quantify the microstructures in EM bioimages.

Image analysis via AI techniques, such as machine learning (ML) and deep learning (DL), has received substantial attention in the fields of biomedicine [397–399] and imaging research [400, 401]. Inductive analysis using supervised data has been used to identify characteristic changes in morphology [402]. Initially, such AI techniques were applied in neuroanatomy research. Kaynig et al. also demonstrated fully automatic 3D segmentation of thin, elongated, cell membrane structures of dendrites for 30 sections in TEM images by finding features from the images and constructing a features classifier using a random forest, a ML method that uses ensemble learning [403]; this tool was supplied as a plug-in of ImageJ/Fiji [404, 405]. Turaga et al. presented a ML algorithm for training a classifier to produce affinity graphs, representing the x-, y- and z-direction information, which can be used to segment the EM images of neural tissue [406]. Subsequently, they reported an affinity graph computation that used a four-layered convolutional neural network (CNN) trained with the supervised dataset; this resulted in 3D reconstructions of neurites with ~90% segmentation accuracy in a 3D EM dataset of rabbit retina tissue [407]. These were the original applications of ML and DL in connectomics studies. Subsequently, AI technologies have become indispensable for bioimage analysis. For instance, DL models, which use an exquisite combination of multi-layered CNN for learning [408, 409], are the current state-of-the-art technology in image recognition [401, 410]; they can extract morphological features, such as the cell body and nucleus, from cellular images via complex networks [411, 412].

For quantification of bioimaging data by DL, the U-Net model was proposed by Ronneberger et al. [413, 414]; the model has encoder and decoder parts with multi-layered CNNs and contracting paths between the encoder and decoder. U-Net targets segmentation tasks in a small number of image datasets with large feature types that are unique to the bioimage dataset. The U-Net model substantially improves performance in segmentation tasks such as cell division tracking and neuronal cell membrane segmentation [414]. Many derivative models, such as FusionNet [415], enhanced U-Net [416], U-Net-Id [417], SCAU-Net [418] and Dual ResUNet [419], have subsequently been proposed that reportedly improve performance compared with that of the original U-Net model. In addition, 3D applicable models have been developed as an extension to 3D volume data [420–422]. Therefore, the U-Net method was a leading technique that has served as a foundation for biological image analysis. For further details, refer to the review by Siddique et al. [423], which comprehensively discusses the U-Net models and derived models.

To use ML and DL techniques universally, user interface tools, such as QuPath [424], Microscopy Image Browser [425], NuSeT [426], UNI-EM [427] and DeepMIB [428], have also been developed. Additionally, the CDeep3M tool can perform image segmentation in cloud computing [429]. A generalist DL model for cellular segmentation, so-called Cellpose, was proposed, which can be precisely applied to the segmentation of cells from a wide range of image types [430]. The DL model has also been ported to R, and typical models for segmentation are available in the ANTsX ecosystem [431]. In addition, we have recently begun distributing supervised bioimage datasets in the R array format that can be used in the analytical workflow in the R environment as the BioImageDbs project via the Bioconductor ExperimentHub platform [432]. Wei et al. introduced the MitoEM dataset, a 3D mitochondria instance segmentation dataset with approximately 40,000 instances and 30 µm3 volumes brain cortices [433].

In contrast to these successes in bioimage data analysis, automated segmentation tasks related to EM images remain challenging because the texture and intensity variation are generally similar [434]. A benchmark report comparing published seven models for EM images
showed that their performances were still highly variable [434]. In recent years, however, successful research cases of EM image analysis using DL have been reported, including in neuroanatomy research and from other fields. In neuroanatomy research, the 3D segmentation of neurites using the DeepEM3D model achieved close to human-level performance [435]. Lee et al. reported a residual symmetric U-Net architecture that achieved an approximately 2%–3% error rate for an EM image dataset of mouse neurites [81] in the SNEMI3D challenge; thus, the system surpassed the human accuracy value provided at that time [436]. Januszewski et al. used a flood-filling network (FFN) to trace neurons in a dataset from a zebra finch brain obtained using SBF-SEM; they achieved high-precision automated reconstruction of neurons with a mean error-free neurite path length of 1.1 mm [437]. Sherida et al. reported that the addition of local shape descriptors promotes affinity-based segmentation methods to a level that is on par with that of the current state-of-the-art system for neuron segmentation based on FFN [438]. Other researchers have attempted segmentation analysis of cell bodies and cell nuclei [251, 390, 439, 440] as well as organelles [441–444] in EM images. For multiple segmentation tasks with EM images, the transfer of learning from pre-trained models using the CEM500K dataset was effective for the transferability of learned features, indicating that a large amount of training data is important for encoding bioimages [445]. Comprehensive quantification of multiple organelles in whole cells using DL segmentation has been reported for serial cross-sections of the mouse liver [446] and for cultured cells [231], which suggests the future possibilities for cell biology that may arise from intracellular morphomics.

Importantly, the DL technique has been applied to more than just quantitative analysis. A recent report described a new data reduction and compression scheme (ReCoDe) that converts the raw data from EM images into 100 times more minor data [447]. Furthermore, image generation models, such as CycleGAN [448, 449], have been applied to EM images as denoising [94, 450–452] and image super-resolution techniques [453–456]. The super-resolution DL technique has been applied to an active acquisition pipeline of SEM imaging [457]. Interestingly, image transformation techniques can also reproduce tissue-stained images from unstained or other stained images [284, 458–462]. To expand limited datasets, various proposals have been made for image data augmentation in DL [463]. In future research, these conversion techniques may also be widely applicable to EM images.

7.4. Deep Learning Applications to Medical and Functional Images

The use of DL in pathology has also advanced remarkably. Using AI to analyse tissue sections is often referred to as computational pathology [397]. Cases of its application have been reported in relation to the pathological diagnosis of various cancer types, e.g. breast cancer [464–466], bladder cancer [419], renal cell carcinoma [467], non-small cell lung cancer [468], skin cancer [469] and gastrointestinal cancer [470], with such techniques potentially increasing the precision of oncology results [471]. For kidney disease assessments, the relationship between renal histology and the prognosis/severity of renal disease has been examined using image recognition and comprehensive segmentation of the constituent tissues of renal samples including human renal biopsies [472–475].

Intriguingly, recent DL results have been associated with biological functions such as the prediction of gene expression patterns [476, 477] and genetic mutations from histological images [478, 479]. Prediction of genetic mutation patterns in lung cancer [468], breast cancer [480] and haematologic cancer [481] has been achieved using morphology image recognition, and this enabled severity classification. Digital imaging studies have been conducted to explore the relationship between histology and gene expression patterns for the prediction of genetic variation associated with tissue morphology [482], the RNA-Seq profiles of tumours [483] and multifactorial site-specific signatures of tissue submitting sites [484] from whole-slide images, as well as diverse molecular phenotypes identified by the expression of immune checkpoint
proteins in tumours [485]. Future developments may lead to the prediction of gene expression at the nano-scale level.

To ensure that DL becomes more widely available, two weaknesses must be addressed. First, the manual annotations required for supervised learning are time consuming and costly. For example, for an EM dataset of 1 million µm³, the cost to manually annotate all images will be 10 million dollars [486]. However, using an unsupervised learning method, which learns from the data without any pre-annotated labels [487], is one means of solving this problem. Although such an approach without annotations is still rare, some studies have included autoencoders for unsupervised learning that have achieved an acceptable performance in histological image recognition [488–490]. Second, the computational process along with the heavy optimisation of many parameters remains a ‘black box’, which is not interpretable by humans. Currently, the appropriateness of a network model can only be evaluated using the numerical value of its prediction performance. One solution is to use human-interpretable image features to predict outcomes [485]. In addition, post-DL analysis should be directed at determining which biologically meaningful information can be extracted from the quantification of morphological features.

8. The Blueprint for Morphomics

The development EM has placed nano-scale imaging data at the centre of morphological analysis. Large 2D and 3D datasets can be acquired with a millimetre-wide range at a nanometre resolution. However, previous morphological studies involving big data analysis have been limited to analysis of the individual dataset, sometimes of a single dataset of normal tissue without any comparison. Although comparisons of several morphological features have been conducted in simple datasets, it is currently challenging to compare across datasets or among unbiased structural features.

Morphomics currently stands at means to create a ‘reference morphology’ or reference EM datasets at an early stage in the development of genomics studies. Only if a solid ‘reference morphology’, or at least a standardised workflow including sample preparation, microscopic settings, data storage, data normalisation and image analysis, is established, will it be possible to conduct larger scale comparative studies that could have major biological implications. The use of bioinformatics methods and imaging databases will accelerate this process. In addition, further multi-omics analysis techniques that can bridge the gap between morphomics and other omics data will be powerful tools. In particular, we expect the development of revolutionary methodologies that combine large-scale EM data analysis techniques with analysis of genomics data such as gene mutation and expression data. By incorporating these new methodologies, the field of pathology is expected to progress rapidly, which might include the identification of previously unknown structures, the quantification of rare events, the reclassification of diseases and the automatic diagnosis of diseases. Furthermore, the amount of data that can be analysed is expected to increase dramatically with the development of automatic AI analysis.

Conclusion

Wide-area 2D EM imaging along with large-scale 3D EM image acquisition and its reconstruction for biological tissues are currently defined as next-generation electron microscopy techniques; however, these tools are now more commonly being used to produce massive morphomics datasets. To maximise the utilisation of morphomics data, the general use of DL methods and post-DL analysis will be essential for comprehensively quantifying cellular morphology. Overall, these advanced techniques can be expected to deepen our understanding of living tissues and cells.
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Conflict of interest
The authors declare that they have no conflicts of interest.

Author contributions
RS and SK designed the contents of the review article. AO provided the EM dataset. RS, KY, AO, MT, MS, YM and SK wrote the manuscript. RS and SK edited the entire manuscript.
Figures and Figure legends

**Fig. 1** Imaging principles in transmission and scanning electron microscopy (TEM and SEM)

(a) TEM captures the forward transmitted electrons through the sample, whereas (b) SEM scans the sample with a narrow beam probe and captures various signals with different energies including the backscattered and secondary electrons.
Fig. 2 2D wide-area electron microscopy images for the kidney and liver of rodents

(a) Wide-area TEM imaging of a mouse kidney sample. Weakly enlarged images (i and ii) show the simultaneous imaging of multiple glomeruli and renal tubules; strongly magnified images (iii and iv) are conventional EM views containing endothelial basement membranes, podocytes, mesangial cells and epithelial cells. (b) Wide-area SEM imaging of a rat liver section via the backscattered electron detection method. The tiled images provide a view of Kupffer cells, hepatocytes and endothelial cells in addition to the hepatic lobule with different magnifications.
Fig. 3 3D volume electron microscopy images obtained using array tomography and SEM

(a) Serial sections of an NB4 cell, i.e. a M3 acute myeloid leukaemia cell line. The shape of the nucleus is highly variable even within a single cell. (b) 3D reconstruction of the NB4 cell using about 130 of the serial sections shown in (a). Nuclei, cell body and mitochondria (high electron density organelles) regions of the cell were segmented. (c) 3D reconstruction of the macula densa in the distal tubules of a mouse kidney glomerulus. Nuclei regions of the macula densa were segmented.
Fig. 4 The principles of three 3D electron microscopy methods: (a) FIB-SEM, (b) SBF-SEM and (c) array tomography

FIB-SEM and SBF-SEM involve scraping the sample surface with an ion beam and a diamond knife built into the equipment, respectively; they facilitate observation of new cross-sections. In array tomography, serial sections are made in advance and then the same position of each section is observed using EM.
Fig. 5 Overview of morphomics analysis of biological tissue using various imaging approaches

Biological systems consist of various molecular components such as genes and transcripts (the genome), proteins, lipids, sugars, amino acids and other metabolic components. Quantitative datasets of comprehensive biometric information are usually treated as omics data in biology or bioinformatics. The resulting pattern of molecular information forms the complex structures of tissue and cells in an organism, which is referred to as the morphological features or biological morphome. The nano-scale organisation of the morphome is accessible using the morphomics approaches, such as a large-scale EM, CLEM combined with light microscopy, and CT method, described in this review.
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## Supplementary Table 1. Representative list of current public electron microscopy datasets

| Data repository | Dataset title                                                                 | Biosamples                                                                 | Dataset ID      | Imaging method                        | Dataset size (format) | DOI or URL                                      |
|-----------------|--------------------------------------------------------------------------------|----------------------------------------------------------------------------|-----------------|----------------------------------------|-----------------------|------------------------------------------------|
| CIL             | Perturbation of the glomerular endothelial surface layer results in albumin filtration | Kidney section (100 nm) of adult *Mus musculus*                           | CCDB:9523       | Mosaic TEM (81x76 tiles)               | 17.7GB (TIFF)         | [https://doi.org/10.7295/W9CCDB9523](https://doi.org/10.7295/W9CCDB9523) |
|                 |                                                                                 |                                                                             | CCDB:9525       | Mosaic TEM (69x95 tiles)               | 19.1GB (TIFF)         | [https://doi.org/10.7295/W9CCDB9525](https://doi.org/10.7295/W9CCDB9525) |
| CIL             | Serial section reconstruction of functionally characterized neurons             | Visual cortex section (45 nm) of adult *Mus musculus* brain                | CCDB:8448       | Serial section TEM (1,153 sections)    | 271GB (tar)           | [https://doi.org/10.7295/W9CCDB8448](https://doi.org/10.7295/W9CCDB8448) |
| CIL             | Ultrastructural Characterization of the Mouse Optic nerve Head and Retina       | Retina section of adult *Mus musculus*                                     | CCDB:7742       | SBF-SEM (206 sections)                 | 22.5GB (tar)          | [https://doi.org/10.7295/W9CCDB7742](https://doi.org/10.7295/W9CCDB7742) |
| CIL             | 3D reconstruction using serial section scanning electron microscopy of the optic nerve head in mouse | Optic nerve head section of adult *Mus musculus*                           | CCDB:8391       | SBF-SEM (724 sections)                 | 136GB (rec)           | [https://doi.org/10.7295/W9CCDB8391](https://doi.org/10.7295/W9CCDB8391) |
| IDR             | Virtual nanoscopy of the mouse glomerulus, mouse embryonic fibroblasts, human dendritic cells and a zebrafish embryo slice | Tissues of *Danio rerio*, *Mus musculus* and *Homo sapiens*                | idr0053-faas-virtualnanoscopy | Mosaic TEM                               |                       | [https://doi.org/10.1083/jcb.201201140.dv](https://doi.org/10.1083/jcb.201201140.dv) |
| IDR             | TEM analysis of SARS-CoV-2 infected intestine organoids                          | SARS-CoV-2 infected human intestine organoids                              | idr0083-lamers-sarscov2 | Mosaic TEM                               |                       | [https://doi.org/10.17867/10000135](https://doi.org/10.17867/10000135) |
| IDR             | Electron Micrographs of the nucleus                                            | Human cultured cell                                                        | idr0086-miron-micrographs | FIB-SEM                                    |                       | [https://doi.org/10.17867/10000141](https://doi.org/10.17867/10000141) |
| IDR             | Scanning transmission electron microscopy of Islets of Langerhans               | Pancreas tissue of human type 1 diabetes                                   | idr0116-deboer-npod | Mosaic STEM                               |                       | [https://doi.org/10.17867/10000168](https://doi.org/10.17867/10000168) |
| EMPIAR          | FIB-SEM of a HeLa cell                                                          | HeLa cell sample                                                           | EMPIAR-10311     | FIB-SEM (1,727 sections)               | 94.0 GB (TIFF)        | [https://dx.doi.org/10.6019/EMPIAR-10311](https://dx.doi.org/10.6019/EMPIAR-10311) |
| EMPIAR          | SARS-CoV-2 infection in human adult lung alveolar stem cells                    | human three-dimensional alveolar type 2 cell cultures                      | EMPIAR-10533     | Mosaic TEM                               | 20.8 GB (TIFF)        | [https://dx.doi.org/10.6019/EMPIAR-10533](https://dx.doi.org/10.6019/EMPIAR-10533) |
| EMPIAR          | SARS-CoV-2 productively infects human gut enterocytes                           | SARS-CoV-2 infected intestine organoids                                    | EMPIAR-10404     | Mosaic TEM                               | 156.2 GB (TIFF)       | [https://dx.doi.org/10.6019/EMPIAR-10404](https://dx.doi.org/10.6019/EMPIAR-10404) |
| Collection | Description | Sample | Accession Numbers | Size | Format | Link |
|------------|-------------|--------|-------------------|------|--------|------|
| EMPIAR | Serial Block Face SEM of HeLa cell pellet with 10 nm pixels and 50 nm slices (benchmark dataset) | HeLa cell sample | EMPIAR-10094 SBF-SEM (518 sections) | 129.8 GB (DM4) | https://dx.doi.org/10.6019/EMPIAR-10094 |
| EMPIAR | SBF SEM images of a Zebrafish hindbrain macrophage containing a replicating Toxoplasma gondii tachozoite | Zebrafish hindbrain sample | EMPIAR-10462 SBF-SEM (557 sections) | 338.4 GB (DM4) | https://dx.doi.org/10.6019/EMPIAR-10462 |
| Nanotomy | Nanotomy of blistering diseases | Human autoimmune blistering disease pemphigus | Mosaic SEM | http://www.nanotomy.org/OA/Sokol2015JD/ |
| Nanotomy | Islets of Langerhans during Type I Diabetes | Pancreas tissue in type 1 diabetic rats | Mosaic TEM | http://www.nanotomy.org/islets2/navigate.html |
| OpenOrganelle | Macrophage cell | Wild-type THP-1 macrophage. THP-1 human monocyte cell line (ATCC TIB-202) treated with PMA to differentiate into macrophages | jrc_macrophage-2 FIB-SEM; Dimensions (µm): 40 x 8 x 37 (x, y, z) | 10.25378/janelia.13123385 |
| OpenOrganelle | Killer T-Cell attacking cancer cell | OT-I mouse cytotoxic T lymphocyte attacking an ID8 cell | jrc_etl-id8-1 FIB-SEM; Dimensions (µm): 74 x 13 x 42 (x, y, z) | 10.25378/janelia.13114454 |
| OpenOrganelle | Immortalized breast cancer cell (SUM159) | Wild-type SUM-159 cell, treated with 0.5 mM oleic acid for 45 mins prior to high pressure freezing to induce the formation of lipid droplets. | jrc_sum159-1 FIB-SEM; Dimensions (µm): 64 x 11 x 35 (x, y, z) | 10.25378/janelia.13114352 |
| BossDB | IARPA MICrONS Pinky100 | High-resolution electron microscopy, segmentation, and morphological reconstruction of cortical pinky100 | Tape-collected section TEM; Dimensions (µm): 499.71 x 335.87 x 87.08 | https://bossdb.org/project/microns-pinky |
| BossDB | Phelps, Hildebrand, & Graham, et al 2021 | A transmission electron microscopy dataset of the ventral nerve cord of an adult female Drosophila melanogaster. | FANC | Tape-collected section TEM; Dimensions (µm): 590.03 x 1140 x 198 | https://bossdb.org/project/phelps_hildebrand_graham2021 |
|---|---|---|---|---|---|
| BossDB | Morgan et al. 2020 | Serial section electron microscopy volume of the mouse dorsal lateral geniculate nucleus (dLGN). | lgn | ATUM-SEM; Dimensions (µm): 800 x 800 x 300 | https://bossdb.org/project/morgan2020 |

Data information was obtained on Sep.–Nov. 2021. Abbreviation: CIL; Cell Image Library, IDR; Image Data Resource, EMPIAR; Electron Microscopy Public Image Archive, and BossDB; Brain Observatory Storage Service & Database.