

Volumetric trans-scale imaging of massive quantity of heterogeneous cell populations in centimeter-wide tissue and embryo

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The **important** study established a large-scale objective and integrated multiple optical microscopy systems to demonstrate their potential for long-term imaging of the developmental process. The **convincing** imaging data cover a wide range of biological applications, such as organoids, mouse brains, and quail embryos, but enhancing image quality can further enhance the method's effectiveness. This work will appeal to biologists and imaging technologists focused on long-term imaging of large fields.

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Abstract

We established a volumetric trans-scale imaging system with an ultra-large field-of-view (FOV) that enables simultaneous observation of millions of cellular dynamics in centimeter-wide three-dimensional (3D) tissues and embryos. Using a custom-made giant lens system with a magnification of 2× and a numerical aperture (NA) of 0.25, and a CMOS camera with more than 100 megapixels, we built a trans-scale scope AMATERAS-2, and realized fluorescence imaging with a transverse spatial resolution of approximately 1.1 μm across an FOV of approximately 1.5 × 1.0 cm². The 3D resolving capability was realized through a combination of optical and computational sectioning techniques tailored for our low-power imaging system. We applied the imaging technique to 1.2 cm-wide section of mouse brain, and successfully observed various regions of the brain with sub-cellular resolution in a single FOV. We also performed time-lapse imaging of a 1-cm-wide vascular network during quail

embryo development for over 24 hours, visualizing the movement of over 4.0×10^5 vascular endothelial cells and quantitatively analyzing their dynamics. Our results demonstrate the potential of this technique in accelerating production of comprehensive reference maps of all cells in organisms and tissues, which contributes to understanding developmental processes, brain functions, and pathogenesis of disease, as well as high-throughput quality check of tissues used for transplantation medicine.

Introduction

Recently, life sciences have focused on comprehending the working principles of multicellular systems, spanning from basic biology to medical applications (1–5). Multicellular systems are generally complex, comprising heterogeneous cells rather than homogeneous assemblies. To understand the mechanism by which numerous cells cooperate to express the function of the entire system, it is ideal to observe all individual components within the system simultaneously during the time span of a whole phenomenon. In particular, in scenarios where a small fraction of cells in a large multicellular system may influence the fate of the population, or when distant cells and tissues operate synchronously, deducing the entire system from observations of sub-populations becomes challenging. Therefore, the trans-scale measurement of collective dynamics of all individual cells that constitute the system of interest is desired.

In response to this demand, researchers have recently reported the development of imaging methods with large field-of-view (FOV) (6–11). Whereas most of these methods are focused on observing brain activity in neuroscience using two-photon excited fluorescence (7–10), our efforts have been dedicated to developing techniques as versatile tools for studying multicellular systems science and developmental biology. In our previous work, we proposed a fluorescence imaging system for the visible wavelength region, capable of spatially resolving individual cells within a centimeter FOV, which we named AMATERAS-1 (A Multi-scale/modal Analytical Tool for Every Rare Activity in Singularity), enabling simultaneous observation of dynamics in the range of 10^5 – 10^6 cells (11). Additionally, we successfully detected less than 1% of cells with unique roles in multicellular systems and revealed how these rare cells trigger a drastic transition from unicellular to multicellular behavior in *Dictyostelium discoideum* (12). However, the imaging system had a numerical aperture (NA) of 0.12, which is insufficient for realizing volumetric observation owing to its broad depth of field (DOF). Overcoming this limitation and enabling volumetric imaging for diverse three-dimensional (3D) tissues and small organisms has been a significant challenge.

In this study, we developed AMATERAS-2, a volumetric optical imaging system with approximately $15 \times 10 \text{ mm}^2$ FOV, equivalent to that of AMATERAS-1 (11). The key component for the enhancement is the giant 2× lens system with an NA of 0.25, which offers improved 3D spatial resolution and higher sensitivity. By incorporating novel methodologies of optical sectioning and computational sectioning, we successfully added volumetric imaging capabilities. The effectiveness of these advancements was demonstrated in imaging 1.5-mm-thick brain section blocks and conducting a 25-h time-lapse observation of vascular endothelial cells during quail embryogenesis, allowing us to trace the spatiotemporal dynamics of approximately 4.0×10^5 cells in three dimensions.

Result

Optical configuration and performance

AMATERAS-1 utilized a telecentric macro lens with 2× magnification, thus allowing dynamic observation in the centimeter-FOV with subcellular resolution (11). Despite its efficacy in biological studies, its low NA (0.12) limits both its spatial resolution in the z-direction, and system brightness. To overcome these constraints and expand the possibilities for observing cell dynamics in tissues and embryos, we developed a giant lens system with a higher NA, consisting of a pair of objective and tube lenses. The objective lens is composed of 12 optical elements in 7 groups, while the tube lens is composed of 9 lenses in 6 groups (Fig. 1A). Both lenses are infinity-corrected with NAs of 0.25 for the objective and 0.125 for the tube lens, and have a field number of 44 mm (diameter) suitable for large-area image sensors. They are effectively aberration-corrected to the diffraction limit or lower in the visible wavelength range of 436–656 nm. The objective lens and tube lens have sizes of 144 mm × 310 mm and 170 mm × 345 mm, respectively (Fig. 1B). The objective and tube lenses exhibit zero vignetting, enabling observation of peripheral areas of the FOV with high brightness.

As the NA of AMATERAS-2 is 0.25, more than double that of AMATERAS-1 (0.12), our system in principle achieves over twice the spatial resolution in the transverse (xy) direction, more than four times the resolution in the longitudinal (z) direction, and over four times the brightness of AMATERAS-1.

Using this new lens system, we constructed a wide-field epi-illumination fluorescence imaging system, termed AMATERAS-2w, with a magnification of 2×, an NA of 0.25, and a field number of 44 mm (Fig. 1C, Materials and Methods). We utilized either a 120-megapixel CMOS camera or a 250-megapixel CMOS camera, depending on the research purpose. Both the cameras have an approximately 35 mm diagonal, thus providing an observation FOV of 17.8 mm and 17.4 mm diagonal at 2× magnification, respectively. The pixel sizes for these cameras are 2.2 and 1.5 μm, with sampling intervals of 1.1 and 0.75 μm at 2× magnification, respectively.

We installed a high-brightness LED light sources for fluorescence excitation, directing it into the objective lens through a custom-made long-pass dichroic mirror designed for GFP imaging with a cut-off wavelength of 497 nm. To ensure uniform illumination across the entire FOV, we employed a lens-let array pair. Additionally, we incorporated a 2-inch band-pass fluorescence filter between the tube lens and the camera.

We experimentally obtained the optical point spread function (PSF) using green fluorescent beads (center wavelength: 515 nm) with a 0.2-μm diameter, employing both the 120-megapixel CMOS camera (Fig. 2A) and the 250-megapixel CMOS camera (Fig. 2B). The left panels in Figs. 2A and B display the PSFs in the xy and xz planes, respectively. The single-point spatial resolution was evaluated by the full-width of half-maximum (FWHM) of the line profiles determined through Gaussian-function fit. The transverse resolutions (xy-direction) obtained with the 120- and 250-megapixel cameras were 1.24 ± 0.12 (N = 100) and 1.12 ± 0.072 (N = 100) (Figs. 2A–B, right top), both of which are slightly downgraded from the theoretical FWHM,

$1.05 \mu\text{m}$, given by $0.51 \lambda_{em}/NA$ ($\lambda_{em} = 515 \text{ nm}$, $NA = 0.25$) (13,14). This can be attributed to the coarse sampling interval (1.1 μm and 0.75 μm, respectively). In particular, in the 120-megapixel case, the apparent single-point resolution varies depending on the relative position of the bead within the pixel (Figs. S1A–B). The coarse sampling also introduces uncertainty in the estimated position of the bead's center coordinate (Fig. S1E). Although this may pose some challenges in quantitatively evaluating fine shapes in cells, it is not a practical issue in imaging at cell resolution. By contrast, the 250-megapixel camera provides a nearly constant resolution (Figs. S1C–D). Thus, the 250-megapixel camera is suitable when high-resolution images are required, whereas the 120-megapixel camera is suitable for other cases where increasing the number of photons per pixel or suppressing data size is necessary.

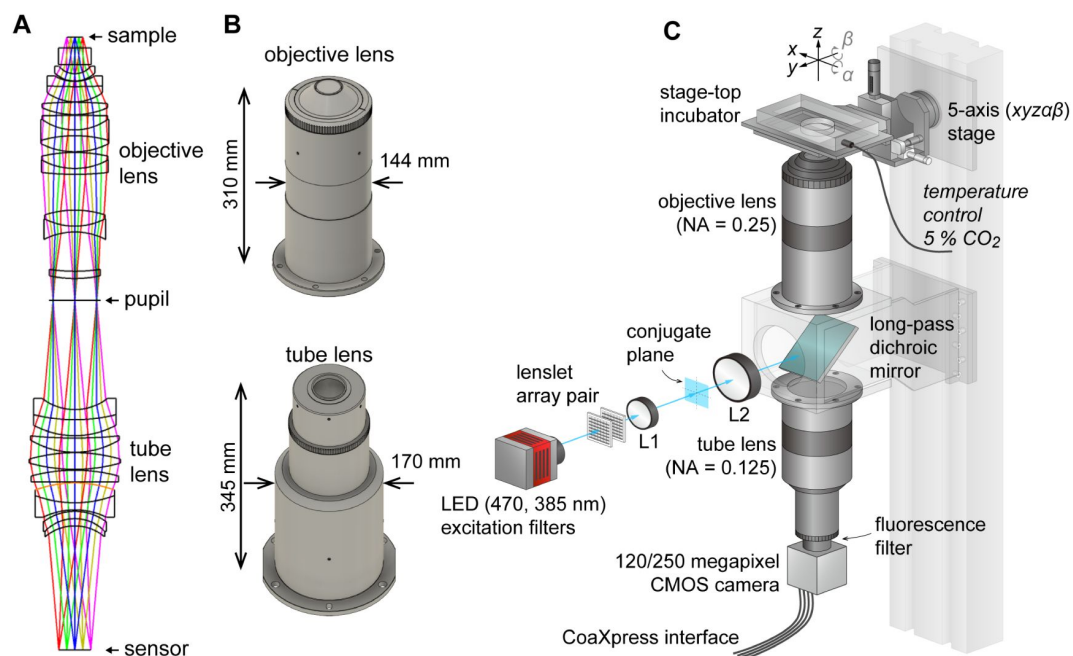


Fig. 1.

AMATERAS-2, a centimeter-FOV cell-imaging system

(A) Schematics of the design of the objective and tube lenses for AMATERAS-2. (B) Appearance of the objective and tube lenses with the width and length. (C) Diagram of the wide-field fluorescence imaging system, AMATERAS-2w, using the two lenses in (A-B). See Materials and Methods for detail.

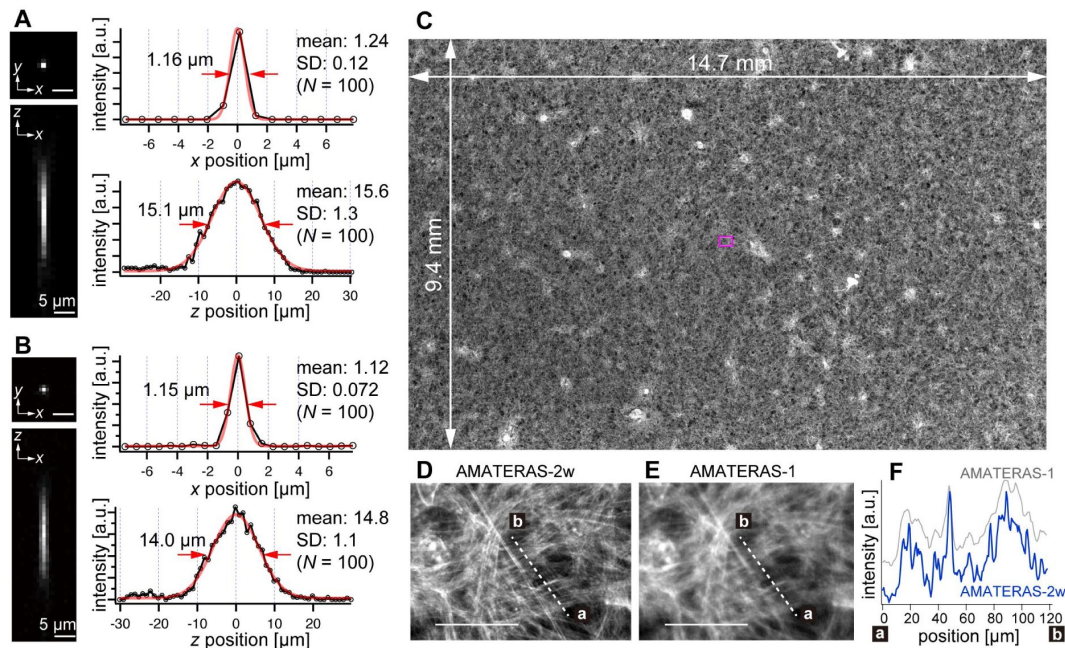


Fig. 2.

Optical performance of AMATERAS-2w

(A),(B) Experimentally obtained PSF of green fluorescent beads with a 0.2-μm diameter in the xy and xz planes, captured using CMOS cameras with (A) 120 megapixels (pixel size = 2.2 μm) and (B) 250 megapixels (pixel size = 1.5 μm). The representative line profiles in the x and z directions on the beads are shown (black lines with circular markers) along with Gaussian fit curves (red lines) and FWHMs, and the mean and standard deviation (SD) calculated for 100 beads are described beside each profile. (C) Fluorescence image of a cardiomyocyte sheet in the entire FOV. (D) Digitally magnified view of the area indicated by a magenta square at the center of (C); scale bar, 100 μm. (E) Same area as (D) but observed by AMATERAS-1 for comparison. (F) Line profiles of the lines in (D) and (E) for comparison of spatial resolution.

In the depth direction (z-direction), we evaluated the DOF by fitting Gaussian curves to the line profiles (**Figs. 2A-B**, right bottom). The DOF was measured to be 15.6 ± 1.3 ($N = 100$) and 14.8 ± 1.1 ($N = 100$) for the 120- and 250-megapixel cameras, respectively. This difference in DOF is attributed to the distinct pixel sizes of the cameras (15). The DOF value for the 250-megapixel camera was found close to the theoretical FWHM, $14.3 \mu\text{m}$, given by $1.78 \lambda_{em}/NA^2$ ($\lambda_{em} = 515 \text{ nm}$, $NA = 0.25$) (14).

The spatial resolution in the transverse direction has been significantly improved, approximately two times compared with that of AMATERAS-1. **Figures 2C and D** display fluorescence images of cardiomyocytes derived from human induced pluripotent stem cells (hiPSCs) stained with Rhodamine phalloidin (C) across the entire FOV and (D) within a central local region indicated by a magenta square in (C). These images were captured using the 250-megapixel camera, and the FOV size was $14.7 \times 9.4 \text{ mm}^2$. For comparison, **Fig. 2E** shows an image of the same area observed with AMATERAS-1. Evidently, AMATERAS-2w delivers more detailed images with finer spatial structures. This is further confirmed by the line profiles depicted in **Fig. 2F**, where the filamentous actin stained with Rhodamine exhibits a sharper spatial resolution with AMATERAS-2w. This result indicated the capability of observing subcellular structures, such as myofibrils, in cell sheets with a large area, such as artificial myocardial sheets, which would enable us to simultaneously investigate microscale structures and macroscale multicellular dynamics.

Spinning pinhole-array disk provides optical sectioning capability in wide-FOV

This imaging system utilizes wide-field illumination and detection, which currently lacks the capability to selectively acquire images of specific z-planes for volumetric imaging. To extend the system's applicability, we developed two methods to enable the volumetric imaging, namely, optical sectioning and computational sectioning.

For optical sectioning, various options of optical techniques exist for achieving 3D fluorescence imaging with a large FOV in the visible wavelength region. These include scanning confocal microscopy (6,13), light-sheet microscopy (4,16), light-field microscopy (17), two-photon excitation microscopy (7–10,18), and spatiotemporal focusing (19). Among these options, we selected the confocal imaging method for this study. This is because the light-sheet microscopy struggles with uniform illumination over the centimeter scale FOV of AMATERAS (16,20), and the light-field microscopy involves a trade-off between spatial resolution and the ability to resolve three dimensions, which is incompatible with the AMATERAS's concept (21). In addition, two-photon excitation microscopy, including that using spatiotemporal focusing, is crucial for deep tissue imaging, but still requires further innovations in pulsed laser power and scanning methods to be effectively applied to the wide FOV of AMATERAS.

We considered the confocal method to be the most compatible with our imaging system. Considering the vast FOV, we adopted the multipoint-scanning confocal system. The commonly used method involving a combination of a microlens array and a pinhole array disk (22) could not be employed in AMATERAS owing to the large NA of the tube lens ($NA = 0.125$) compared with that of standard microscopes. Matching the NA requires shortening the distance between the two disks or enlarging the microlenses. However, shortening the inter-disk distance leads to a loss of space for inserting a dichroic mirror, and enlarging the microlenses reduces the number of foci. While confocal reflection (non-fluorescence) microscopy does not require a beam splitter, allowing for close microlens-pinhole spacing (23), the confocal fluorescence microscopy requires a beam splitter due to the essential separation of fluorescence and excitation light. Therefore, designing microlens-pinhole arrays suitable for AMATERAS-2 presents a significant challenge. This challenge can be circumvented by using an optical configuration where excitation light and fluorescence pass through synchronized pinhole arrays on separate paths, as initially proposed in the early development of multipoint confocal microscopy (24). However, this

solution would require duplicating the optics of the giant lens, which is currently unfeasible. Moreover, even if possible, the added complexity of the system would render this approach impractical.

To address this challenge, we opted to use pinhole arrays without microlenses, which is rather a more traditional configuration of multipoint confocal microscopy (13,25,26). Although this method is less light-efficient compared with the one using microlenses, it aligns well with our imaging system's high NA and low magnification. Here, we designed the pinhole array disk for fluorescence imaging in green (*e.g.*, GFP, YFP) and red (*e.g.*, RFP, mCherry) wavelengths. Commercial confocal systems using pinhole-array disks typically feature large pinhole sizes (*e.g.*, 50 μm) and wide spacing between pinholes (*e.g.*, 200 μm), designed for general microscopes. For AMATERAS-2, however, with its 2 $\times$ magnification and NA of 0.25, the pinhole size must be much smaller. The diameters of the Airy disk for green and red wavelengths are about 5 μm and 6 μm , respectively, at the focal plane of the tube lens ($\text{NA} = 0.125$). As a prototype, we set the pinhole size to 6 μm (Fig. 3A), matching the Airy disk diameter for red wavelength. A more detailed discussion on the impact of pinhole diameter dependence on 3D resolution is available in the Supplementary File (Note 1, Fig. S2). The pinhole spacing was set as 24 μm , striking a balance between throughput and crosstalk (Fig. 3A). To implement this, we custom-made a pinhole-array disk and incorporated it into a rotary machine (CrestOptics, Rome, Italy).

We positioned the pinhole array disk at the image plane of the tube lens and constructed a relay lens system to transfer the plane to the camera. This particular configuration, termed AMATERAS-2c, is illustrated in Fig. 3B (Materials and Methods). To split the light paths of fluorescence excitation light, we placed a short-pass dichroic mirror beneath the disk. For fluorescence excitation, we used a high-brightness LED with a center wavelength of 470 nm, the same as in the wide-field imaging system (Fig. 1C). The relay lens consisted of a telecentric macro lens with 1 $\times$ magnification, and a 2-inch band-pass fluorescent filter was positioned at the entrance of the macro lens. If necessary, the relay lens can be replaced by a 2 $\times$ magnification lens to switch the total magnification from 2 $\times$ to 4 $\times$, based on resolution requirements.

Fluorescence images of 0.5 μm fluorescent beads were observed with and without the pinhole-array disk, where fluorescent beads were three-dimensionally dispersed in an agarose gel slab (Fig. 3C). The presence of the pinhole-array disk dramatically suppressed background light from outside the focal plane. This verified that optical sectioning can be achieved by the pinhole-array disk. The variations in the 3D PSF with and without the pinhole-array disk are detailed in the Supplementary File (Fig. S3). Typical 3D PSF was obtained with the fluorescence beads with the relay lens of (D) 1 $\times$ and (E) 2 $\times$ magnifications (Figs. 3D and E). As NA of the 1 $\times$ relay lens ($\text{NA} = 0.079$) is lower than that of the tube lens ($\text{NA} = 0.125$), the transverse spatial resolution (Fig. 3D, right top) is degraded compared to the one obtained by the wide-field configuration without the relay lens (Figs. 2A and B, Note 1). By contrast, the 2 $\times$ relay lens ($\text{NA} = 0.12$) almost matches with the tube lens, and the reduction in the transverse spatial resolution is relatively small (Fig. 3E). As for the longitudinal resolution, the FWHM of the PSF was approximately 16 μm with a 1 $\times$ relay lens and approximately 14 μm with a 2 $\times$ relay lens, both values significantly exceeding the ideal value about 9.5 μm (Note 1). These discrepancies are attributed to the relatively loose focusing of the excitation light and the non-negligible size of the pinhole.

Computational sectioning provides pseudo-depth-resolved imaging capability

In addition to optical sectioning, we developed a computational sectioning method to separate images of the focal plane and out-of-focal planes. During measurement, a z-stack is acquired in the wide-field imaging configuration (Fig. 1C) by scanning the focal position in the z-direction and capturing images at each step. The raw image in a 3D volume results from the superposition of in-focus and out-of-focus plane images. We estimated the contribution from out-of-focus planes as the

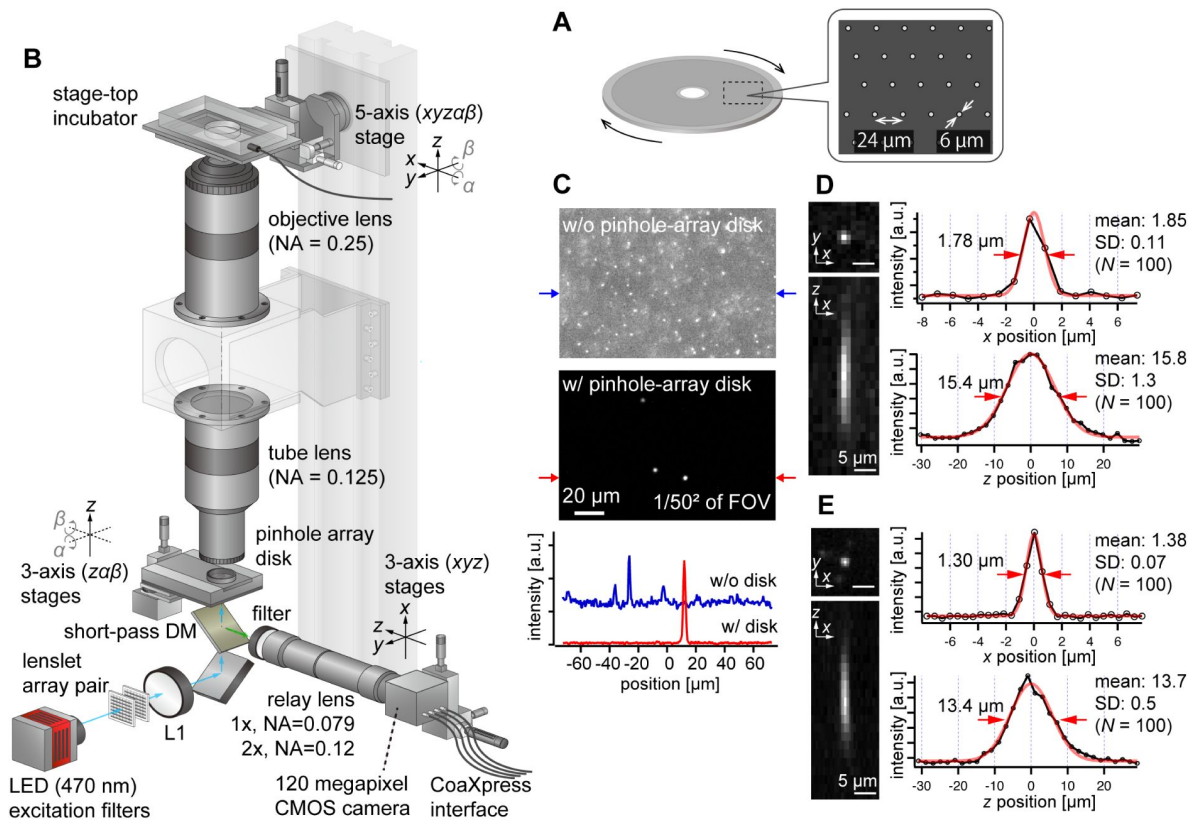


Fig. 3.

AMATERAS-2c, a multipoint confocal imaging system for 3D volume imaging.

(A) Design of our pinhole-array disk. (B) Schematic of the optical configuration using the pinhole-array disk. (C) Effect of background light rejection by the presence of the pinhole-array disk in a $292 \times 202 \mu\text{m}^2$ area at the FOV center corresponding to $1/50^2$ of the entire FOV. (D),(E) Experimentally obtained PSF of green fluorescent beads with a diameter of $0.5 \mu\text{m}$ in the xy and xz planes, captured using relay lens with (D) $1\times$ magnification (NA = 0.079) and (E) $2\times$ magnification (NA = 0.12). The representative line profiles in the x and z directions on the beads are also shown (black lines with circular markers) along with Gaussian fit curves (red lines) and FWHMs, and the mean and standard deviation (SD) calculated for 100 beads are described beside each profile.

baseline by iteratively applying low-pass filtering under the assumption that the image's spatial frequency in the focal plane is higher than that in out-of-focus planes. The cut-off frequency of the low-pass filter was optimized using the principles of independent component analysis (Materials and Methods, Note 2, Fig. S4-S5).

To demonstrate computational sectioning, we observed myocardial organoids derived from hiPSCs (27 [↗](#)). This 3D dome structure of a cavity chamber is extensively studied in developmental biology and regenerative medicine as a model for human myocardial tissue development (28 [↗](#), 29 [↗](#)). We fabricated a large area of organoids across the entire FOV, chemically fixed and immunostained them for cardiac troponin T (cTnT) with Alexa488, and stained the nuclei with Hoechst33342 (Materials and Methods). The z-stack was obtained within a z-range involving the organoids (approximately 250 μm) with 4- μm intervals. **Figures 4A and B** [↗](#) display two-color overlaid images of the island-formed organoids across the entire FOV, without and with computational sectioning, respectively (both are maximum-intensity projection (MIP) images of the z-stacks). **Figures 4C and D** [↗](#) present images of six layers in the z-direction of the yellow square region of **Figs. 4A and B** [↗](#), before and after computational sectioning, respectively. After the sectioning, the distribution of cTnT and nuclei in each z-layer is clearly visualized (**Fig. 4D** [↗](#)): The island areas are composed of multilayered cells, and the inter-island spaces are covered with a single layer of cells, as shown in **Fig. 4D** [↗](#), $z = 20 \mu\text{m}$. A 3D isosurface representation (**Fig. 4E** [↗](#)) shows that the hollow oval structure is formed by a thin layer of cont.-positive cells (cardiomyocytes) whereas the underlying cell layer is composed of cTnT-negative cells, consistent with previous literature (27 [↗](#)). This demonstration validates the technique for wide-field imaging. Video files of **Figs. 4C-E** [↗](#) are available in the Supplementary files (Movies S1 and S2).

In principle, the signal component can be extracted as long as the signal intensity (in-focus component) is significantly higher than the statistical noise of the background intensity (out-of-focus component). This method is effective in cellular imaging when fluorescent molecules are localized within the cell, like in the nucleus, or when they exhibit a filament-like structure, thereby enabling cell recognition and dynamic tracking. It relies on the spatial frequency of the fluorescence image in the focal plane being uniform and clearly higher than that outside the focal plane.

Observing a 1.2-cm wide and 1.5-mm thick volume of mouse brain section

To demonstrate the potential for brain imaging, we performed imaging of a 1.5-mm thick mouse brain section in the coronal plane (**Fig. 5A** [↗](#)). The brain was chemically cleared using CUBIC (30 [↗](#), 31 [↗](#)), and the cell nuclei were stained with SYTOX-Green (Materials and Methods). The 1 $\times$ relay lens was primarily employed (total magnification = 2 $\times$) to cover the coronal plane in the FOV. Each layer's exposure time was 1 s, and a total of 378 layers were acquired to cover the entire 1.5-mm-thick volume in steps of 4 μm in the z-direction. Optical and computational sectioning were both applied. **Figure 5B** [↗](#) shows a fluorescence image at a single z-layer ($z = 700 \mu\text{m}$), revealing the 12 mm $\times$ 8 mm wide section with single cell resolution. **Figure 5C** [↗](#) displays enlarged views of the xy-plane (coronal plane) in the dotted square region in **Fig. 5B** [↗](#), along with xz (transverse plane) and yz (sagittal plane) cross sections, essentially demonstrating successful z-direction sectioning. **Figure 5D** [↗](#) shows a 3D representation of the same volume of raw data obtained by optical sectioning alone, prior to computational sectioning. However, owing to intense background light overlapping, individual cells are challenging to distinguish, particularly in regions of high cell density. By utilizing computational sectioning to eliminate background light, clear images were obtained even in areas with strong background light. Supplementary files contain videos of the z-scan for the entire FOV and local regions (Movies S3 and S4).

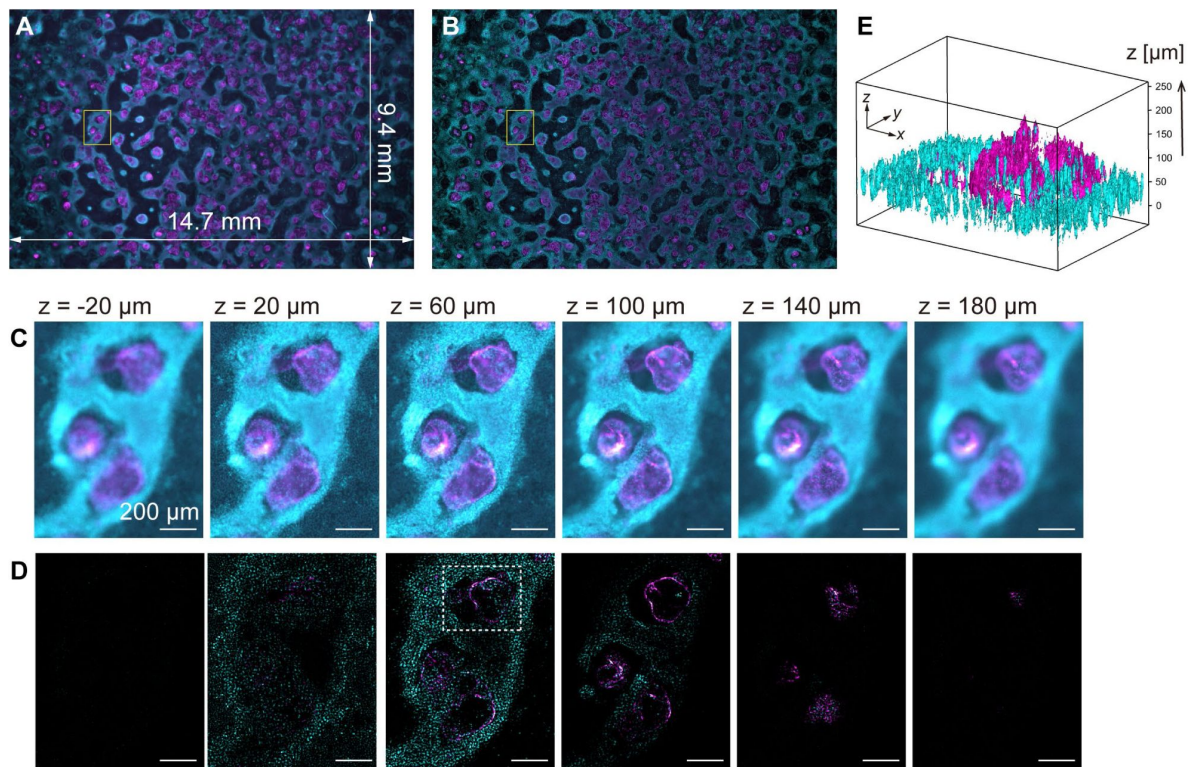


Fig. 4.

Volumetric imaging with computational sectioning.

(A),(B) Two-color images of the cavity chamber structure of a myocardial organoid (A) before and (B) after computational sectioning. Both (A) and (B) are MIP images of the z-stack data, captured using the 250-megapixel CMOS camera; here, magenta and cyan represent the distribution of immunostained cardiac troponin T and Hoechst-labeled nuclei, respectively. (C),(D) Six z-layers with a difference of 40 μm in the yellow square region indicated in (A),(B); scale bars, 200 μm . (E) Three-dimensional isosurface representation in the dashed square region indicated in (D).

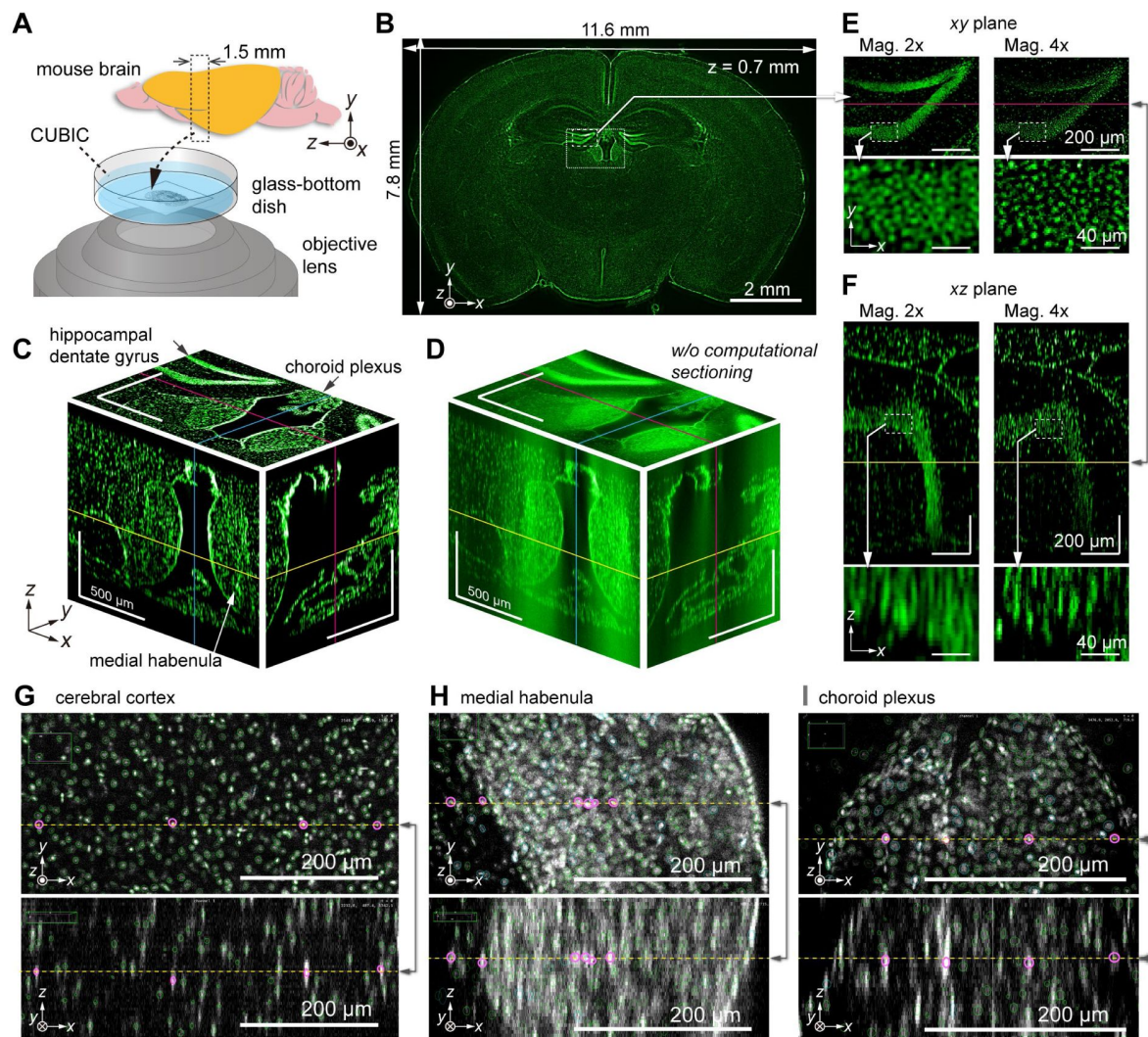


Fig. 5.

Volumetric imaging of a mouse brain section.

(A) Schematic of a mouse brain showing the placement of the brain sample in the inverted configuration. (B) Schematic of a single z-layer in the coronal plane with size of the effective area covering the entire brain section. (C) Fluorescence images of three orthogonal cross sections of the dotted square indicated in (B); here, the yellow, cyan, and magenta lines represent the z-, x-, and y-positions of the cross sections, respectively. (D) Cross-sectional images of raw data in the same region as (C), where the computational sectioning is not applied. (E),(F) Magnified images of the hippocampal region (dashed square in (B)) in the (E) xy-plane and (F) xz-plane. For comparison, images obtained with 2× (left) and 4× (right) magnification systems are shown together. Note that the 3D regions observed at different magnifications do not perfectly match owing to the difficulty in optical alignment. (G-I) Cell detection by ELEPHANT in three brain regions, the cerebral cortex (G), medial habenula (H), and choroid plexus (I), where pairs of intersecting xy-and xz-planes are arranged vertically. The detected cells within each plane are marked with green and cyan ovals, and the straight line shared by the two planes is represented by a yellow dashed line, with certain cells along this line highlighted by magenta ovals. The xy-planes overlay the oval markers within a range of $\pm 25 \mu\text{m}$ above and below the focal plane, while the xz-plane shows only oval markers on the plane.

Figure 5C visualizes the characteristic 3D structures of several regions, including hippocampal dentate gyrus, medial habenula, and choroid plexus, which are known to be associated with memory formation (32), depression (33), and regulation of cerebrospinal fluid (34), respectively. Close-up views of the hippocampal region (indicated with the dashed square in **Fig. 5B**) in the *xy* and *xz* planes are shown in **Figs. 5E and F**, respectively. A comparison of the images at 2× and 4× magnification in **Figs. 5E and F** reveals that the higher magnification (4×) allows for improved spatial resolution and separation of individual cells in both the *xy* and *xz* planes.

For cell detection in the 3D imaging data of the mouse brain section, we used a recently developed interactive platform for cell detection and tracking, called ELEPHANT (Efficient LEarning using sParse Human Annotations for Nuclear Tracking) (35). This platform seamlessly integrates manual annotation with deep learning and allows for result proofreading (Materials and Methods). Using this advanced image analysis technique, cell nuclei were detected within the 3D volume. **Figure 5G** illustrates the cell detection results on specific *xy* and *xz* planes within the cortex. As the cell density in the cortex is relatively low, the ellipsoid-shaped nuclei image does not overlap with neighboring cells, resulting in the successful detection of nearly all cells (as clearly shown in the *z*-scan video, Movie S5). The precision and recall of the cell detection were estimated to be 99.4 % and 97.6 %, respectively (Materials and Methods). Based on these results, the cell density in the cortex was calculated to be 1.18×10^5 cells/mm³, which closely matches previous reports (36). This technique was also applied to two other brain regions, the medial habenula and choroid plexus (**Figs 5H-I**). In these regions, cell density is higher than in the cortex and the signal-to-noise ratio is lower due to strong background intensity, making it difficult for traditional methods to detect cells accurately. However, with the assistance of ELEPHANT, we successfully detected the majority of cells.

Dynamics of over a 4.0×10^5 cells were captured over 24-hours development of a quail embryo

We employed the imaging methods described for a time-lapse observation of cell migration during quail development. The non-confocal optical configuration (AMATERAS-2w, **Fig. 1C**) and utilizing computational sectioning alone were sufficient for this 250-μm thick sample. We used a tie1:H2B-eYFP transgenic quail embryo (37) in which enhanced yellow fluorescent protein (eYFP) visualizes the nuclei of vascular endothelial cells (Materials and Methods). The embryo was cultured *ex ovo* on a 35 mm glass-bottom dish (**Fig. 6A**). Time-lapse fluorescence imaging began at 36 h (HH10) after the start of egg incubation and captured eYFP signals in the developing embryo over 24 h. For 3D imaging, we acquired *z*-stacks every 7.5 min, with each *z*-stack containing 21 layers spaced 12 μm apart. This resulted in a total of 4200 image layers and 200 time-points, with a data size of approximately 500 GB. During the time-lapse observation, we stabilized the relative distance between the lens and the sample using a self-developed autofocus system (Materials and Methods).

Representative images of cell distribution based on the nuclear eYFP signals at four time-points (*t* = 0, 8, 16, and 24 h) are shown in **Figs. 6B and C**. These images were reconstructed using the HSB color model to represent the 3D distribution after the baseline was removed by computational sectioning (Fig. S6). The brightness indicates the intensity of the MIP image in the *z*-direction, whereas the hue represents the *z*-position with the maximum value in the MIP process (Materials and Methods). As observed in **Figs. 6B and C**, the cell nuclei distribution undergoes significant changes over time, thus resulting in the formation of organs such as the ventral aortae, heart, and dorsal aortae (*t* = 24 h, **Fig. 6C**). The heart region appears blurred owing to its oscillating shape caused by beating. Supplementary files include videos of the HSB-color images in the entire FOV and magnified local regions (Movies S6–S8). **Figure 6D** shows enlarged views of the ventral aortae (indicated by the left dashed square in **Fig. 6C**) at the four time-points. Additionally, a 3D isosurface of the *z*-stack of the dorsal aorta region (the right dashed square in

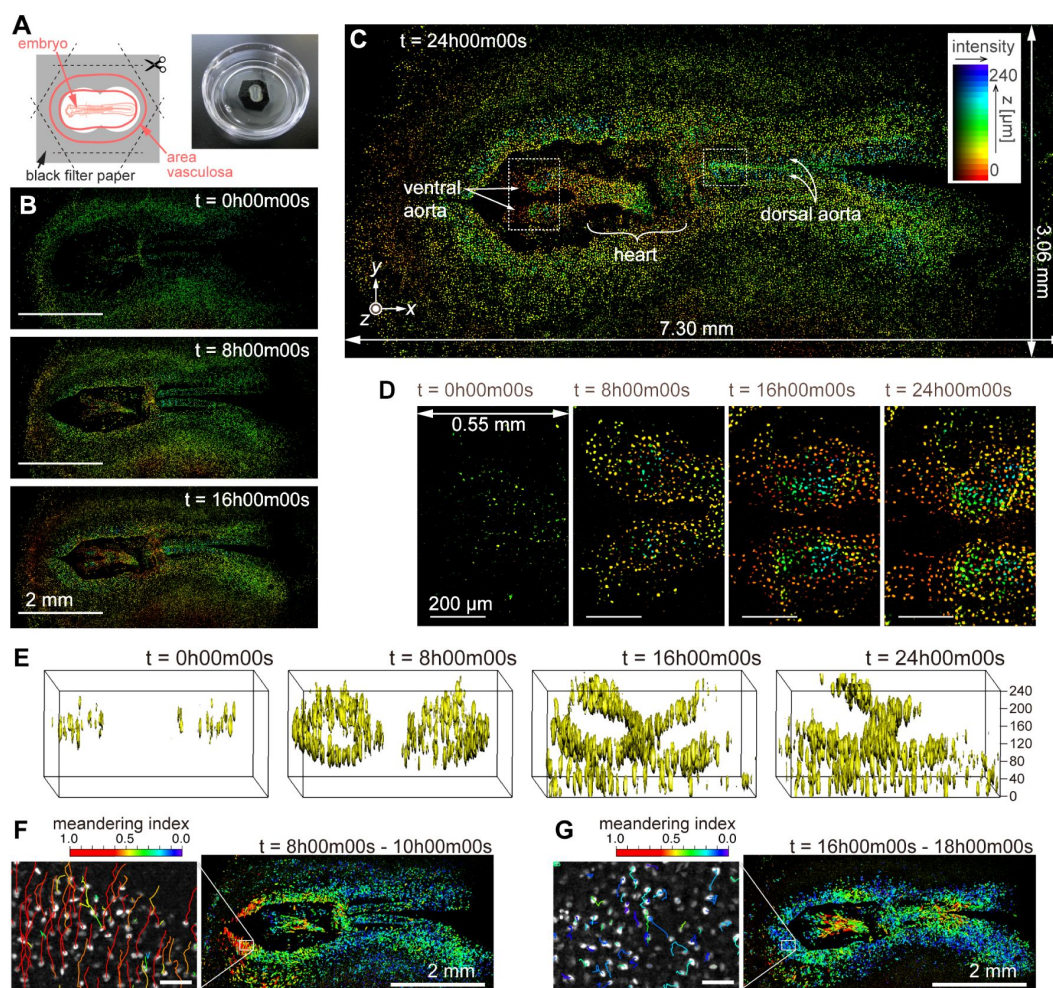


Fig. 6.

Dynamics of vascular endothelial cells in quail embryo captured by time-lapse observation.

(A) Schematic showing how the specimen is mounted (left) and a photograph of a specimen placed in a glass-bottom dish. (B) Representative images obtained at $t = 0$ h, 8 h, and 16 h; scale bars, 2 mm. (C) Enlarged view of the image corresponding at $t = 24$ h; the pseudo-colors represent the z -position of the cells and fluorescence intensity by their hue and brightness, respectively. (D) Magnified views of the anterior side of ventral aortae (left dashed square in (C)) at the four time-points with intervals of 8 h; scale bars, 200 μ m. (E) Three-dimensional isosurface representation of the dorsal aorta region (right dashed square in (C)) viewed from the left side. (F),(G) Cell position trajectories and spatial mapping of features related to the cell dynamics in the two time-regions of (F) $t = 8$ –10 h and (G) $t = 16$ –18 h. Left panels show enlarged views in the white square regions ($292 \times 202 \mu\text{m}^2$) indicated in the right panels. In both the panels, the rainbow-colored trajectories of the cell movement are overlaid on the grayscale MIP images at the first frame in the time regions (F: $t = 8$ h, G: $t = 16$ h). The scale bars are 50 μ m. The rainbow color table represents the meandering index of the cell movement in the two time-regions.

Fig. 6C [↗](#)) was calculated (**Fig. 6E** [↗](#), and Movie S9), clearly showing the developmental process of the two tube structures of the dorsal aortae ([38](#) [↗](#)). Note that the upper vessel wall is partially missing in the second half of the movie because the fluorescent image was disturbed by the blood flow that began around $t = 15$ h.

At the current spatiotemporal resolution, we successfully traced the movement of individual cells. Segmentation of cell nuclei allowed us to detect approximately 4.5×10^6 cells across all 200 frames, and by linking cells in close proximity over consecutive frames, we tracked about 4.0×10^5 cells. **Figures 6F and G** [↗](#) show cellular movement along with a feature parameter related to cell movement at $t = 8$ h and 16 h. Both left panels are magnified views of the square regions in the right panels, with trajectories drawn on MIP images. By analyzing the cell segmentation and tracking results, various feature parameters related to cell nucleus morphology and dynamics were computed, including nuclear size, brightness, aspect ratio, velocity, acceleration, mean square displacement, and meandering index ([39](#) [↗](#), [40](#) [↗](#)). Here, we used the meandering index, a measure representing the straightness of cellular movement, to color the trajectories in **Figs. 6F and G** [↗](#) with the rainbow color table. We found that the distribution of high meandering index at $t = 8$ h and 16 h was significantly changed. Further detailed analysis and biological interpretation employing the cell-tracking results will be discussed in a future paper.

Discussion

In this study, we presented AMATERAS-2w, a large FOV fluorescence imaging system, along with its variant AMATERAS-2c. The system is built around a newly designed giant lens with an NA of 0.25, magnification of 2 $\times$, and field number of 44 mm. This innovative lens configuration allows for imaging with an impressive spatial resolution of approximately 1.1 μm within a centimeter-scale FOV (15 $\times$ 10 mm²). Notably, this FOV-to-spatial resolution ratio surpasses those of previously reported large-FOV imaging methods. The key metric "optical invariant", derived from the product of NA and FOV, has a value of 2.75 (Materials and Methods, 41), thus indicating excellent performance of the lens system. Furthermore, the "space-bandwidth product", which signifies the FOV to spatial resolution ratio achieved under real measurement conditions (considering image sensor size and wavelength), reaches 4.3×10^8 (Materials and Methods, 42). Both of these metrics significantly outperform those reported in earlier studies on large FOV imaging methods ([6](#) [↗](#)–[10](#) [↗](#), [41](#) [↗](#)). Although these metrics are essential indicators of our system's performance, they do not solely determine its superiority over other large-FOV imaging systems. Variations in biological targets and additional optical performance measures (such as frame rate and imaging depth) must also be considered. Our primary focus in designing this system was to expand the FOV, i.e., maximize the number of observable cells at any instant, even at the cost of intracellular spatial resolution. Thus, in this aspect, our system excels as an instrument for such purposes.

The spatial resolution (FWHM) of AMATERAS-2w and -2c was measured to be approximately 1.1–1.8 μm in the transverse direction (xy) and 13–16 μm in the longitudinal direction (z) through PSF measurements (**Figs. 2A** [↗](#), **2B** [↗](#), **3D** [↗](#), and **3E** [↗](#)). These values represent a significant enhancement compared with the previous AMATERAS version (2.3 and 64 μm , respectively) ([11](#) [↗](#)). The improved transverse resolution enables clearer visualization of intracellular filamentous structures (**Fig. 2F** [↗](#)). Moreover, the refinement in longitudinal resolution was more than four times greater, thus demonstrating that the expected effect was achieved with more than double NA. Depending on the object of observation, it may be debatable whether this anisotropic spatial resolution elongated along the optical axis qualifies "cellular resolution". Although this may be insufficient to resolve dense cell populations, it proves adequate for cells with only stained nuclei or those sparsely distributed, as observed in **Figs. 5F–I** [↗](#), and **6E**. Using modern cell detection methods compatible with 3D big data, such as ELEPHANT employed in this study ([35](#) [↗](#)), we can achieve accurate cell detection, counting and tracking to some extent, even without clear spatial separation in three dimensions. It goes without saying that the higher spatial resolution in

raw image data would enhance the performance of subsequent image analysis. The next step in optical development involves further enhancing the NA of the lens system, including the relay lens, to further improve volumetric resolution and expand the range of observable biological objects.

We successfully achieved volumetric imaging of a 1.5-mm-thick mouse brain coronal section using the multipoint-scanning confocal imaging method, along with computational sectioning and tissue-clearing (**Fig. 5**). This thickness corresponds to about one-tenth of the whole brain. The exposure time per image was 1 s, and data acquisition took less than 10 min. The ultimate goal is to apply this approach to enable whole-brain imaging. Unlike previous methods such as light-sheet imaging (30) and block-face serial microscopy tomography (43), which require half a day or more for whole-brain data acquisition owing to tiling images from a narrow FOV, our method can significantly reduce the acquisition time when extended to the whole brain. This feature is effective not only for the brain imaging but also for imaging of other organs and whole body of an organism. We also plan to apply it to live imaging of highly transparent tissues such as zebrafish (44) and organoids (45). Nevertheless, as the thickness increases, challenges such as increased background light and larger spherical aberration arise, thus resulting in weaker focal plane images. To address these issues and realize whole-brain imaging, we propose the incorporation of a mechanism for compensating spherical aberrations in the future. Additionally, we aim to improve the pinhole array pattern design and light source intensity to enhance transmittance and increase fluorescence intensity from samples, broadening the scope of applications.

We have shown that AMATERAS-2 can perform time-lapse observation of the centimeter-sized vascular networks in the quail embryo. It is a promising tool for understanding the multicellular behavior, especially the mechanisms of tissue formation during development (1–3). In regard to the quail embryo's case, the data obtained here are the first demonstration of the dynamics of the extensive organization of the vascular network throughout the quail embryo at the single-cell level. Conventional methods of research employing traditional microscopy have selected a limited area for observation and thus had a difficulty in determining the distribution and synchronicity of cellular dynamics across the entire system. By analyzing such a huge four-dimensional data (xyz and t), we can gain insight into when and where each organ is formed, in what order, and in what interrelationships. In addition, employing this method allows us to break away from the conventional hypothesis-driven research manner and obtain new findings in a data-driven manner. This advantage can be realized not only for quail embryo but also for a variety of other species undergoing widespread and dynamic cellular events, making it a powerful tool in the study of multicellular organisms, especially in the study of tissue formation.

To further advance multicellular systems research, information on individual cell states is required in addition to cellular dynamics, therefore multiplex measurement is an essential technological challenge. Although most of the observations presented in this paper were made with monochromatic fluorescence imaging, multiplex imaging will lead to a more profound understanding if information on the distribution of cellular states can also be obtained by employing multiple fluorescent probes. This requires technological upgrades in optical filters and light sources for multicolor imaging. In addition, spatial transcriptomics has been rapidly developing in recent years and has become one of the most crucial tools for multicellular system study (5). In the near future, dynamics imaging and spatial transcriptomics for the same sample will be a very important technological integration to advance an integrative understanding of self-organization in tissue formation. Rather than simply using AMATERAS sequentially with existing spatial transcriptomics, it could be employed as an optical detection system for spatial transcriptomics to achieve extremely high throughput in the same wide FOV of cellular dynamics imaging.

Finally, let us discuss a practical challenge we encountered with our instrument, primarily related to the handling of extensive image data. In our studies, the raw data for 3D imaging of the mouse brain section and time-lapse 3D imaging of the quail embryo amounted to approximately 100 and 500 GB, respectively. However, after multiple image processing and analysis steps, a single experimental measurement resulted in several terabyte of image data. Managing such vast data proves challenging for standard computers in terms of both software and hardware capabilities. To address software limitations, we opted not to employ commercial software for data analysis. Instead, we utilized our originally developed programs, implementing certain optimizations to reduce analysis time, minimize write/read cycles, and prevent memory overflows. As for hardware, we established a data server with petabyte storage, thereby enabling efficient data sharing among collaborators from diverse research institutions physically distant from one another. The existence of this infrastructure considerably facilitated the smooth progress of collaborative research within this study. Considering the increasing significance of handling image big data, we expect methodologies for image computation to become even more critical in the future. Ideally, a comprehensive system should be developed, encompassing not only data storage and sharing but also an integrated analysis solution in the cloud. We would like to actively promote the development of such a system, essentially encompassing advancements in imaging techniques and data-handling strategies.

Materials and methods

Wide-field imaging system (AMATERAS-2w) configuration

We aimed for AMATERAS to achieve a larger FOV-to-resolution ratio than conventional microscopes, which required a giant imaging lens system. We realized this design concept by collaborating with SIGMAKOKI CO., LTD. Inc. (Tokyo, Japan) to manufacture the giant objective and tube lenses as shown in [Fig. 1](#). The characteristics of these lens are described in the main text. Designing such large lenses presents significant challenges in selecting optical glass materials compared to regular-sized lenses. Although there are over 200 types of optical glass available, our options are limited because the thickness or diameter of several types of glass may be unprocessable, or their transparency may degrade due to their size. Therefore, we iteratively explored the optimal design, considering the feasibility of material procurement and processing in conjunction with the optical design.

By combining these lenses, we constructed an imaging system with a 2× magnification, NA of 0.25, and a 44 mm field number. To facilitate both water-immersion and dry observation, we incorporated a glass plate whose thickness can be adjusted at the lens tip. The objective lens has a focal length of 120 mm, whereas its working distance is 14 mm owing to the presence of the attachment at the tip. The pupil positions of the objective lens and the tube lens are set externally to the lens bodies. This arrangement allows for the placement of a spatial filter or other devices at the pupil position, facilitating future enhancements in functionality.

For image acquisition, a 120-megapixel CMOS camera (VCC-120CXP1M, CIS, Tokyo, Japan) and a 250-megapixel CMOS camera (VCC-250CXP1M, CIS, Tokyo, Japan) were used, out of which we selected one depending on research purpose. Both the cameras have a chip size of 35 mm (diagonal). The pixel sizes of these two cameras are 2.2 μm and 1.5 μm, respectively. The sampling intervals are 1.1 μm and 0.75 μm at 2× magnification. Image data captured by the CMOS cameras are loaded into a workstation via a CoaXpress frame grabber board (APX-3664G3, Aval Data, Tokyo, Japan). After the imaging experiments, they were transferred to a network server with large storage capacity for long-term storage and data sharing among project members.

By use of the lens system and CMOS cameras, a wide-field epi-illumination fluorescence imaging system was constructed. The imaging system chassis was designed to accommodate both inverted and upright microscope geometries. For this study, all experiments were conducted using the inverted microscope arrangement. Epi-illumination was achieved using a high-brightness LED sources (SOLIS-470C and SOLIS-385C, Thorlabs, Newton, NJ), with an excitation filter (#87-800, Edmund Optics, Barrington, NJ) placed immediately after it. The illumination light was homogenized using a pair of lenslet arrays and projected onto the sample surface. Additionally, a similar LED light source (SOLIS-525C, Thorlabs, Newton, NJ) with an illumination homogenizer was also mounted as transmitted illumination light for bright-field observation. To split the light path, we used a custom-made long-pass dichroic mirror measuring 158 mm × 120 mm × 10 mm (BK7), which was designed for fluorescence imaging of GFP with a cut-off wavelength of 497 nm. A fluorescent filter (#86-992, Edmund Optics, Barrington, NJ) was positioned in front of the camera, which was firmly attached to the imaging lens using an F-mount.

The sample stage comprises a three-axis translational movable stage and a two-axis tilt stage. Only the z-axis stage is motorized, utilizing an electric actuator (SOM-C13E, SIGMAKOKI CO., LTD., Tokyo, Japan). For time-lapse observation under controlled conditions, we mounted a stage-top incubator (SV-141A, BLAST, Kawasaki, Japan) on the five-axis stage, providing a stable environment at 37 °C with CO₂ control. To ensure precise measurements, we enclosed the entire sample space within a dark box, shielding it from external light and temperature fluctuations.

Multipoint confocal system (AMATERAS-2c) configuration

In the multipoint confocal imaging system, we utilized a custom-made pinhole array disk with specific dimensions (pinhole size: 6 μm, spacing: 24 μm). This disk was mounted on a high-speed rotating machine (CrestOptics, Rome, Italy) and placed precisely on the image plane of the imaging lens. Alignment throughout the FOV was ensured using a translation stage (XR25P/M, Thorlabs, Newton, NJ) and tilt stage (AIS-1016B, SIGMAKOKI CO., LTD., Tokyo, Japan) to adjust height and tilt. Beneath the disk, we positioned a short-pass dichroic mirror (DMSP490L, Edmund Optics, Barrington, NJ) to allow excitation light to be incident from below. The excitation light source included a high-brightness LED (SOLIS-470C, Thorlabs, Newton, NJ), an excitation filter (#87-800, Edmund Optics, Barrington, NJ), and a pair of lenslet arrays (#63-231, Edmund Optics, Barrington, NJ), together with a 75-mm lens (*f* = 200 mm, LA1353-A, Thorlabs, Newton, NJ) to ensure uniform illumination onto the disk. The fluorescent image was reflected by the dichroic mirror, transmitted through a fluorescent filter (#86-992, Edmund Optics), and projected onto a 120-megapixel camera using either a 1× or 2× relay lens (LSTL10H-F and LSTL20H-F, Myutron, Tokyo, Japan). The relay lens and camera were attached with an F-mount and secured on a three-axis translational stage (PT3/M, Thorlabs, Newton, NJ). To adjust the focus, the stage for the optical axis direction was motorized using an electric actuator (SOM-C25E, SIGMAKOKI CO., LTD., Tokyo, Japan).

Measurement of 3D point spread functions

To evaluate the optical performance of the imaging systems, the 3D point spread function was measured using green fluorescent beads. Fluorescent beads with a 0.2 μm diameter (FluoSphere F8811, Invitrogen, ThermoFisher Scientific, Waltham, MA) were employed in the evaluation of AMATERAS-2w (Figs. 2A-B [↗](#)) and those with a 0.5 μm diameter (FluoSphere F8813, Invitrogen, ThermoFisher Scientific, Waltham, MA) were employed in the evaluation of AMATERAS-2c (Figs. 3D-E [↗](#)). The beads were dispersed three-dimensionally in agarose gel on a glass-bottom dish, and z-stacks were obtained by moving the sample in the z-direction in 1 or 2 μm steps.

Computational sectioning

In wide-field fluorescence imaging of a 3D volume, the superimposition of a fluorescent image from the focal plane and background light from outside the focal plane is a fundamental issue. This problem is not limited to wide-field imaging but also arises in multipoint confocal imaging of

thick or high-density samples. To overcome this challenge, we employed computational sectioning, an image processing technique aimed at removing the background light component. Various algorithms have been proposed (46 [DOI](#), 47 [DOI](#)), and Leica microscopes' standard software includes this functionality. In this study, we developed an original algorithm as a simple and robust method for data analysis.

The critical step in our approach involves estimating the background light component at each layer of the z-stack data. To achieve this, we leverage the assumption that the background light exhibits a low spatial frequency, whereas the focal plane image demonstrates a high spatial frequency. Consequently, we employ an iterative low-pass filtering technique to estimate the background light component. Specifically, as shown in Figs. S6A and B, a two-dimensional (2D) low-pass filter is applied to the raw data ($f_0(x,y)$) to obtain a smooth surface ($L_0(x,y)$). To obtain $f_1(x,y)$, $f_0(x,y)$ and $L_0(x,y)$ are compared and the smaller one is chosen to $f_1(x,y)$ at every position (x,y) , as expressed by Eq. (1 [DOI](#)).

$$f_j(x, y) = \min \left(f_{j-1}(x, y), L_{j-1}(x, y) \right) \quad (1)$$

where $\min(a,b)$ returns the smaller value of a and b . Next, the low-pass filter is applied to $f_1(x,y)$ to obtain $L_1(x,y)$; this process is repeated to make $L_j(x,y)$ closer to the baseline of the original image. The iteration stops when the standard deviation of the difference of $f_j(x,y)$ and $L_j(x,y)$ reaches a preset value (ε).

$$\sqrt{\text{Var} \left(f_j(x, y) - L_j(x, y) \right)} < \varepsilon \quad (2)$$

Among various methods available as 2D low-pass filters, we sequentially applied one-dimensional (1D) infinite impulse response (IIR) filter in two directions (x,y). This method is faster than other methods such as a 2D Fourier transform (48 [DOI](#)). This speed difference is especially significant when the number of pixels in an image is massive, as in the present case. We adopted the Butterworth type of the IIR low-pass filter.

When the focal plane is empty and there is a bright object in the background, the baseline estimation tends to be skewed by the intensity fluctuations of the background. To mitigate this, we applied a 3×3 moving average filter to the entire image as a pre-processing step before initiating the baseline estimation algorithm. A critical parameter in our algorithm is the cutoff frequency of the low-pass IIR filter. If the cutoff frequency is set too high, the focal plane component would be included in the background; if it is set to low, background light would remain in the focal plane. Thus, it is crucial to find an optimal middle ground for the cutoff frequency. During this optimization, we did not account for cell size or optical system performance. Indeed, we employed a user-friendly blind separation method based on independent component analysis (ICA) to enhance usability (49 [DOI](#)). Similar to ICA, we assumed that the fluorescence image in the focal plane deviates from the Gaussian (normal) distribution and that the superposition of images from multiple planes, including both in-focus and out-of-focus planes, results in a distribution closer to the Gaussian distribution. To quantify the non-Gaussian nature of the distribution, we considered several measures, including kurtosis, skewness, negentropy, and mutual information (49 [DOI](#)). Among these measures, we found skewness to be the most robust metric for our image dataset and incorporated it into our algorithm. The cutoff frequency was adjusted to maximize the skewness of the estimated in-focus image. The optimization of the cut-off frequency was performed on a subset of the data before applying it to the entire dataset. An example of the selection of non-Gaussianity measure and the optimization of the cut-off frequency is found in the Supplementary File (Fig. S4-S5, Note 2). The typical cut-off frequency was set at 0.06 in normalized frequency unit of 1/pixel, and the value was applied across all data.

Culture of hiPSCs and differentiation into cardiac organoid with cavity chamber structure

A hiPSC line 201B7 was routinely maintained as previously described (50) on iMatrix-511 (Matrixome, 89292) coated culture dish in StemFit medium (Ajinomoto, AK02N), and 10 μ M Y-27632 (Fujifilm Wako, 030-24021) was added for the first 24 h after passage. Four days before inducing differentiation, hiPSCs were dissociated with TrypLE Select Enzyme (Thermo Fisher Scientific, A1285901) and 1.9×10^5 cells were passed into iMatrix-511 coated 12 well plate in StemFit medium supplemented with 10 μ M Y-27632. After 24 h, the medium was changed to StemFit without Y-27632 and was exchanged daily for 3 days. Differentiation was initiated with the CDM3 medium (51) composed of RPMI-1640 with HEPES (Fujifilm Wako, 189-02145), 0.5 mg/ml human recombinant albumin (Sigma Aldrich, A9731) and 0.2 mg/ml L-ascorbic acid 2-phosphate (Sigma Aldrich, A8960) supplemented with 3 μ M CHIR99021 (Fujifilm Wako, 038-23101) for 48 h. On day 2 of differentiation, the medium was changed to CDM3 supplemented with 5 μ M IWP-2 (Fujifilm Wako, 034-24301). From day 4 to day 8, the cells were cultured in CDM3 and the medium was changed every other day. From day 8, the medium was changed to RPMI-1640 with HEPES supplemented with 2% B27 (Thermo Fisher Scientific, 17504044) and was changed every other day. The cells were fixed in 4% paraformaldehyde (PFA; Thermo Fisher Scientific, 43368) on day 15 for immunocytochemical analysis.

Immunocytochemical staining of cardiac organoid

Cells were fixed with 4% PFA for 15 min at room temperature (RT $\sim$ 23 $^{\circ}$ C), permeabilized with 0.2% Triton X-100 (Fujifilm Wako, 807423) for 20 min at RT and incubated with blocking solution composed of D-PBS with 5% BSA and 10 % FBS for 30 min at RT. Subsequently, the cells were incubated with primary antibody (1:200 rabbit IgG anti-cTnT, Bioss, bs-10648R) at 4 $^{\circ}$ C overnight. After washing three times, cells were incubated with secondary antibody (1:500 alexa488 conjugated goat IgG anti-rabbit IgG, Abcam, ab150077) for 2 h at RT. The cells were washed three times in D-PBS with 1 μ g/ml Hoechst33342 (Dojin, H342). Subsequently, the cells were incubated with D-PBS and used for fluorescent observation.

Color representation of the 3D image

Figures 6B–D show the 3D positions of the cells using the HSB (also known as HSV) color model. After applying the method described earlier to remove background light from the z-stack image, we conducted a maximum value intensity projection along the z-direction. The z-position of the maximum value at each xy-position was then recorded (Fig. S6C). The color image was reconstructed by associating the brightness and hue of the HSB table with the value of the maximum intensity and the maximum value position, respectively (Fig. S6D). For the hue table, we assigned red to blue to z-layer numbers ranging from 0 ($z = 0 \mu$ m) to 20 ($z = 240 \mu$ m).

Preparation of mouse brain section

All animal care and handling procedures were conducted with approval from the Animal Care and Use Committee of Osaka University (approval numbers R02-8-7). Our utmost efforts were made to minimize the number of animals used. Experiments involved adult male C57BL/6J mice (SLC, Shizuoka, Japan) aged between 2 and 3 mo. To ensure proper anesthesia, mice were deeply anesthetized through intraperitoneal injection of an anesthetic cocktail containing 0.3 mg/kg medetomidine (Nippon Zenyaku Kogyo, Fukushima, Japan), 4 mg/kg midazolam (Sandoz Pharma, Basel, Switzerland), and 5 mg/kg butorphanol (Meiji Seika Pharma, Tokyo, Japan). Subsequently, anesthetized mice underwent transcardial perfusion with saline, followed by 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) dissolved in phosphate-buffered saline (PBS). Brain tissues were then excised and immersed in a 4% paraformaldehyde solution until further use. For tissue preparation, brain tissue blocks were sliced into 1.5-mm thick coronal sections

using a vibrating microtome (LinearSlicer Pro7N, Dosaka EM, Kyoto, Japan). Subsequent staining and tissue clearing involved immersion of sections in CUBIC-L (Tokyo Chemical Industry, Tokyo, Japan) for delipidation, SYTOX Green (Thermo Fisher Scientific, Waltham, MA, USA) solution (1:5,000) diluted in 20% (vol/vol) DMSO in PBS for nuclear staining, and CUBIC-R+(M) (Tokyo Chemical Industry, Tokyo, Japan) for refractive index matching, following previously established methods (31 [↗](#), 52 [↗](#)). After clearing, tissue sections were placed on glass bottom dishes and embedded in 2% (wt/vol) agarose gel prepared with CUBIC-R+(M) for subsequent imaging analysis.

Preparation of quail embryo

Transgenic quail line, tie1:H2B-eYFP (37 [↗](#)) was bred in quail breeding facility at Kyushu University. Fertilized eggs were incubated at 38 °C. The staging of quail embryos was based on the Hamburger and Hamilton stages of chicken embryos (53 [↗](#)). The animal study was approved by the Institutional Animal Care and Use Committee of Kyushu University (Authorization number: A20-019). *Ex ovo* culture was performed as previously described (54 [↗](#)). Black filter paper was used instead of white filter paper to avoid fluorescence background.

Autofocus in time-lapse imaging

To address z-directional drift in the time-lapse observation of quail, we developed an original autofocus method. During the intervals between fluorescence z-images, we acquired bright-field z-stacks and analyzed the images to identify the most in-focus position. To achieve this, we used a black ink marker located on the substrate edge as the autofocus target instead of the variable sample itself. For evaluating the degree of in-focus, we applied a total variation (TV) filter to the marker image and used the kurtosis of the filtered image as the focus score. The z-position with the maximum focus score was identified as the in-focus location, thus signifying the sharpest image of the marker edge. Although other filter types and statistical moments are available options for the focus score (55 [↗](#)), our preliminary experiments demonstrated the suitability of the kurtosis of TV for the marker image. In the actual experiment, we acquired 15 images in 15 μm steps and plotted the focus scores against z-positions. Through quadratic function fitting of the focus scores at three z-positions (including the z-position with the maximum value and adjacent positions), we estimated the best in-focus position with an accuracy of 1 μm.

Deep-learning based cell detection for the mouse brain data

Cell detection was conducted using ELEPHANT, a unified platform that facilitates manual annotation, deep learning and proofreading of results within a single user-friendly GUI (35 [↗](#), 56 [↗](#)). ELEPHANT serves as an extension to Mastodon (57 [↗](#)), an open-source framework for large-scale tracking deployed in Fiji, a widely used image-analysis software (58 [↗](#)). ELEPHANT supports incremental deep learning with sparse annotation, incorporating algorithms for detecting cells in 3D and tracking them in time-lapse 3D image datasets. In this paper, we utilized the cell detection function exclusively for analyzing 3D imaging data of mouse brain section (Fig. 5G-I [↗](#)). The incremental learning approach allows cell detection models to be trained progressively on a dataset that begins with sparse annotations and is continuously enhanced through human proofreading. For visualization, image data were displayed using BigDataViewer (59 [↗](#)) on Mastodon, accessed via BigDataServer (60 [↗](#)). This setup permits the visualization of large-scale image data on client computers while maintaining the data on the server. Deep learning capabilities were provided by the ELEPHANT server, enabling remote GPU access. This setup allows non-experts to perform image analysis on big data using deep learning, effectively overcoming typical challenges such as the need for large amounts of high-quality training data, the absence of an interactive user interface, and limited access to computing resources, including substantial storage and high-end GPUs (61 [↗](#), 62 [↗](#)). The server computer used in this study is equipped with an Intel(R) Xeon(R) Gold 6132 CPU @ 2.60GHz, runs Ubuntu 20.04, and includes 384

GB DDR4 2,666 MT/s RAM, 2x NVIDIA Tesla V100-PCIE-32GB GPUs, and a network-attached Lustre parallel file system with over 2 PB of storage. The client computer features an Apple M1 Pro CPU, runs Sonoma 14.3, and has 16 GB of LPDDR5-6400 RAM and a 500 GB SSD.

In the analyses presented in this paper (**Fig. 5G-I**), cell detection models were trained for three regions, including the cortex. In each region, the detection model was refined by repeating the sequence (annotation, training, prediction and proofreading) five to seven times. By annotating approximately 100 cells in total, we were able to establish a model capable of detecting the vast majority of the cells. The training of the detection models was conducted on volumes of $256 \times 256 \times 16$ voxels, which were prepared by preprocessing with a random flip in each dimension. During the label generation step, the center ratio was set to 0.4 and the background threshold was set to zero, meaning that only explicitly annotated voxels were used for training. In the prediction step, volumes cropped to dimensions of $700 \times 700 \times 100$ around the target area were used as input. In the postprocessing step, a threshold for nucleus center probabilities was set to 0.5, and r_{min} , r_{max} and d_{sup} were set to 1 μm , 5 μm , and 3 μm respectively (see details about the parameters in 35).

To quantitatively evaluate the performance of cell detection, we calculated precision and recall, defined as $TP/(TP+FP)$ and $TP/(TP+FN)$, respectively, where TP stands for true-positive, FP for false-positive, and FN for false-negative. In the actual 3D data of the cortical region, the values of TP, FP, and FN were manually counted by comparing the detection results of cells ($N \sim 640$) within a defined volume of $310 \times 170 \times 100$, one by one, with the actual images. As a result, we obtained a precision of 99.4 % and a recall of 97.6 %.

Cell segmentation and tracking for the quail embryo data

For the analysis of quail embryo data, cell detection, segmentation and tracking were performed using a custom-made program coded in Python. The observed 3D volume has a transverse area (xy) of more than 1 cm^2 and a height (z) of 240 μm , which is relatively thin in the z direction compared to the xy plane. The overlap of multiple vascular endothelial cells in the z direction occurs with a very low probability. Therefore, to save computational costs and time, 2D cell detection was conducted on z -projected MIP images instead of full 3D cell detection. To avoid miss-detection of overlapping cells, we divided the z -stack data into three blocks along the z -axis and created MIP images for each block, on which 2D cell detection was performed on the MIP images. The resulting cell lists were compared to identify identical cells detected across multiple blocks. By recognizing double-detected cell pairs as identical, we updated the cell list accordingly. This process was applied to all double-detected cells, thus achieving effective cell detection in the 3D volume and enabling cell tracking throughout the observation period.

Evaluation of optical system with optical invariant and space-bandwidth product

We employed two indices to evaluate the lens system's scale range and compare it with previous research. The first index, the optical invariant, measures the lens system's performance and is obtained by multiplying the FOV radius and NA. The second index, the space-bandwidth product, considers the image sensor and wavelength while quantifying the ratio of actual resolution to FOV. These indices are calculated using the following simplified formulas, respectively (41).

$$I = \frac{NA \times FN}{2M} \quad (3)$$

$$SBP = 8 \left(\frac{R_{FOV}}{d_{xy}} \right)^2 \quad (4)$$

where I , FN , M , SBP , R_{FOV} , and d_{xy} denote optical invariant, field number, magnification, space-bandwidth product, FOV radius, and spatial resolution (FWHM).

Data and materials availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or Supplementary Materials.

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Author contributions

Conceptualization: TI, TN

Optics design: TI, HH, TN, YT, SE

Lens design and fabrication: YT

Optical system construction: TI, YT, SE

Development of imaging system software: TI

Sample preparation: TK, YS, KS, HH

Data acquisition: TK, YS, TI

Data analysis and visualization: KI, TI, TK, KS

Setup of deep-learning platform: KS, HI, SO

Construction of data share server: HI, SO

Writing—original draft: TI

Writing—review & editing: TI, TN

Competing interests

T.I, H.H and T.N have patent applications (2021-104163(JP), 2023-077341). K.S. is employed part-time by LPIXEL Inc. All other authors declare no additional conflict of interests.

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Reviewer #1 (Public review):

Summary:

The authors are trying to develop a microscopy system that generates data output exceeding the previous systems based on huge objectives.

Strengths:

They have accomplished building such a system, with a field of view of 1.5x1.0 cm² and a resolution of up to 1.2 μ m. They have also demonstrated their system performance on samples such as organoids, brain sections, and embryos.

Weaknesses:

To be used as a volumetric imaging technique, the authors only showcase the implementation of multi-focal confocal sectioning. On the other hand, most of the real biological samples were acquired under the wide-field illumination, and processed with so-called computational sectioning. Despite the claim that it improves the contrast, sometimes I felt that the images were oversharpened and the quantitative nature of these fluorescence images may be perturbed.

<https://doi.org/10.7554/eLife.93633.2.sa2>

Reviewer #2 (Public review):

Summary:

This manuscript introduced a volumetric trans-scale imaging system with an ultra-large field-of-view (FOV) that enables simultaneous observation of millions of cellular dynamics in centimeter-wide 3D tissues and embryos. In term of technique, this paper is just a minor improvement of the authors' previous work, which is a fluorescence imaging system working at visible wavelength region (<https://www.nature.com/articles/s41598-021-95930-7>).

Strengths:

In this study, the authors enhanced the system's resolution and sensitivity by increasing the numerical aperture (NA) of the lens. Furthermore, they achieved volumetric imaging by integrating optical sectioning and computational sectioning. This study encompasses a broad range of biological applications, including imaging and analysis on organoids, mouse brains, and quail embryos, respectively. Overall, this method is useful and versatile.

Weaknesses:

What is the unique application that only can be done by this high-throughput system remains vague. Meanwhile, there are also several outstanding issues in this paper, such as the lack of technical advances, unclear method details and non-standardized figures.

Comments on revisions:

The revised manuscript has significantly improved in response to the initial review comments, particularly with the detailed additions regarding the objective lens and confocal imaging modes, which enhance the clarity and comprehensibility of the paper. While the structure and arguments are much clearer overall, there are still key issues that need to be addressed, specifically regarding algorithm validation, computational sectioning presentation, and volume imaging rate.

Algorithm Validation:

The validation of the algorithm's accuracy is not sufficiently robust. Reviewer 1's comment is entirely reasonable, and the authors should validate the algorithm's accuracy using well-established methods as ground truth. In the revised version, the authors attempt to demonstrate the fidelity of the algorithm by employing deep learning methods for high-accuracy cell recognition. However, this validation relies solely on comparisons between deep learning results and manual annotation results. The problem lies in the fact that both manual annotations and deep learning outcomes are derived from algorithm-processed data, which fails to prove the authenticity or validity of the data itself. To strengthen the validation, the authors should incorporate independent, gold-standard methods for comparison.

Computational Sectioning:

In the revised manuscript, the authors effectively demonstrate the ability of optical sectioning to improve axial resolution using fluorescent beads, as shown in Fig. S3, which is a strong point. However, the manuscript lacks a direct comparison for computational sectioning and does not provide a clear evaluation of axial resolution before and after applying computational sectioning. While some related information is included in Figs. 5.C and D, the details are insufficient, and intensity profiles are absent. I recommend that the authors include more direct visual demonstrations of computational sectioning, along with comparisons of axial resolution before and after applying computational sectioning. This would better showcase the method's effectiveness.

Volume Imaging Rate:

The manuscript currently omits critical details about the method's volume imaging rate. In the description of the quail embryo imaging experiment, key parameters such as exposure time and imaging speed are missing. Additionally, the manuscript does not discuss the maximum imaging rate supported by the system in confocal mode. The volume imaging rate is an essential factor for biological researchers to evaluate the applicability of the technique. Therefore, this information should be included, ideally in the abstract and introduction. Furthermore, the authors could describe how the volume imaging rate performs under different conditions and discuss its potential applications across various biological research contexts. Including such details would significantly enhance the paper's utility and appeal to the broader research community.

These adjustments will further strengthen the manuscript and address the reviewers' concerns effectively.

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Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

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In this study, the authors enhanced the system's resolution and sensitivity by increasing the numerical aperture (NA) of the lens. Furthermore, they achieved volumetric imaging by integrating optical sectioning and computational sectioning. This study encompasses a broad range of biological applications, including imaging and analysis of organoids, mouse brains, and quail embryos, respectively. Overall, this method is useful and versatile.

Weaknesses:

The unique application that only can be done by this high-throughput system remains vague. Meanwhile, there are also several outstanding issues in this paper, such as the lack of technical advances, unclear method details, and nonstandardized figures.

Here, we address the first part of the Weaknesses concerning the unique application, and will respond to the latter part in the Reply to the Recommendations.

We are developing 'large field of view with cellular resolution' imaging technique, aiming to apply it to the observation of multicellular systems consisting of a large number of cells. Our proposed optical system has achieved optical performance that enables simultaneous observation of more than one million cells in a single field of view. In this paper, we have succeeded in adding three-dimensional imaging capability while maintaining the size of this two-dimensional field of view. By simultaneously observing the dynamics of a large number of cells, we can reveal spatio-temporal sequences in state transitions (pattern formation, pathogenesis, embryogenesis, etc.) in multicellular systems and discover cells that serve as a starting point. These were mentioned in the 1st and 2nd paragraphs of the Introduction section (Line 48-, 58-) and discussed in the 4th paragraph of Discussion section (Line 398-) of the main text. While our previous work on two-dimensional specimens has shown its validity, the present work demonstrated that temporal changes of multicellular systems in three-dimensional specimens can be observed at the single-cell level.

Ideally, we aim to achieve the same level of depth observation capability as the FOV size in the lateral direction. However, at present, the penetration depth for living specimens is limited to a few hundred micrometers due to non-transparency, while the lateral FOV size exceeds 1 cm. The current optical performance is well-suited for systems where development occurs within a thin volume but a large area, such as the quail embryo presented in this paper (Fig. 6 in the revised manuscript). In addition to quail embryos, this technique can also be applied to the developmental systems of highly transparent model organisms, such as zebrafish. Furthermore, for chemically cleared specimens, even those thicker than 1.5 mm, as shown in this paper (Fig. 5 in the revised manuscript), can be observed. Besides organs other than the brain, it could also be applied to imaging entire living organisms. However, for observation depths up to 10 mm, such as in the whole mouse brain, a mechanism to compensate for spherical aberration is required, which we consider the next step in our technological development.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

(1) I suggest that authors shall re-examine the quantitative nature of their image processing algorithm. Also, I wonder whether there are parameters that could be adjusted, as images in Figure 3D and 4E seem to be oversharpened with potential loss of information.

As the reviewer pointed out, we recognized that there was an insufficient explanation of the image processing.

Therefore, descriptions on the quantitative nature and parameter adjustments have been added to the text (Materials and Methods, Line 552) and the Supplementary File (Fig. S4-5, Note 2), and these have been referenced in the main text. A summary is given below.

The adjustable parameters in our method include the cutoff frequency of the smoothing filter used in the background light estimation. If the cutoff frequency is too high, the focal plane component will be included in the "background"; if it is too low, background light will remain in the focal plane. The cutoff frequency needs to be optimized within this range. In this optimization, neither the size of the cell itself nor the performance of the optical system was considered; instead, we utilized the concept of independent component analysis (ICA). This approach is taken because the size and structure of cells vary from sample to sample, and the optical properties also vary with wavelength and location, making it impractical to consider

each factor for every case. ICA employs a blind separation method, which is based on the principle that individual signals deviate from the normal (Gaussian) distribution, while the superimposition of signals tends to bring the distribution closer to the Gaussian distribution. Several indices have been proposed to quantify the non-Gaussian nature of the distribution, including kurtosis, skewness, negentropy, and mutual information. Among these measures, we empirically found skewness to be the most suitable and robust, and therefore adopted it for our algorithm. The optimal parameters were selected using a subset of the data before applying the calculations of the entire dataset. The determined values were then applied to the entire dataset.

Regarding the oversharpening, we believe that it rarely occurs in the image data shown in the manuscript. In a case where low-frequency structures and high-frequency structures are mixed in the focal plane, oversharpeninglike effect can occur because of the disappearance of low-frequency structures, which is discussed in Supplementary File (Note 2, Figs. S5D). However, in the case of a sample with nearly uniform spatial frequency, such as the nucleus observed in this study, oversharpening is unlikely to occur by setting appropriate parameters as described above. If it appears that some images are oversharpened in the figures, it is due to the contrast of the image.

(2) On the other hand, I am curious how a wide-field fluorescence system may reliably extract information from a densely labeled sample within axial volume of 200 μm , as they showed in the mouse brain in Figure 4. Thus I am skeptical regarding the fidelity and completeness of the signals and cells recorded there. It would be ideal if the authors could benchmark their system performance with a two-photon microscope system, which serves as the ground truth.

The reviewer's suggestion is reasonable; however, we are unfortunately unable to observe the same sample using a two-photon microscope. Instead, we will explain these differences from a theoretical perspective. Two-photon microscopes used for brain imaging typically employ objective lenses with a numerical aperture (NA) of at least 0.5, allowing for 3D imaging with depth resolution ranging from several micrometers down to sub-micrometer levels. In contrast, our method uses a lens system with NA of 0.25, and the optical configuration (focusing NA, pinhole size) are not optimized for resolution (Note 2 in Supplementary File), thus the longitudinal resolution (FWHM) is about 14 microns (Fig. 3E in the revised manuscript). This difference is significant in the brain imaging, where our method may not fully separate all cells in close proximity along the depth axis, as shown in the bottom panels (xz-plane) of Fig. 5F of the revised manuscript. Nevertheless, we believe that cell nuclei can be accurately detected in this 3D image using appropriate cell detection methods based on deep learning. To support this claim, we conducted cell detection using the state-of-the-art cell detection platform ELEPHANT and incorporated the results into Fig. 5 (Fig. 5G-I). This figure demonstrates that even with the current spatial resolution, accurate detection of cell nuclei is achievable.

We accordingly added one paragraph (Line 285) in the main text to explain the cell detection method and discuss the results. We also added one section into Materials and Methods for more detail of the cell detection (Line 650).

In conjunction with the revision, the developer of ELEPHANT (K. Sugawara) has been included as a co-author.

Reviewer #2 (Recommendations For The Authors):

In my opinion, the following concerns need to be addressed.

Major comments:

(1) The proposed system's crucial element involves the development of a giant lens system with a numerical aperture (NA) of 0.25. However, a comprehensive introduction and explanation of this significant giant lens system are missing from the manuscript. I strongly suggest that the authors supplement the relevant content to provide a clearer understanding of this integral component.

A detailed description of the giant lens system has been added to the main text (Optical Configuration and Performance, Line 83) and the Materials and Methods section (Wide -field imaging system (AMATERAS-2w) configuration, Line 446). A diagram of the lens configuration has also been included in Fig. 1A. In conjunction with these additions, two engineers from SIGMAKOKI CO. LTD., who made significant contributions to the design and manufacturing of the lens system, have been included as co-authors.

(2) The manuscript introduces a computational sectioning technique, based on iteratively filtering technology. However, the accuracy of this algorithm is not sufficiently validated.

It is challenging to discuss accuracy of the processing results compared to the ground truth, because the ground truth is unknown for any of the experiments. Instead, in the Supplementary File (Notes 2, Figures S4-5), we show how the processing results for the measured and simulated data vary with the parameter (cutoff frequency), illustrating the characteristics of our method. The results suggest that by optimally pre-selecting the parameter, it is possible to successfully separate the in-focus and out-of-focus components. This discussion is related to our response to the first recommendation made by the reviewer #1. Please review our response to Reviewer #1 regarding parameter optimization and oversharpening. Here, as an addition, we describe a discussion of the conditions that must be met in order to perform the calculation correctly, as described below (also included in Note 2, Limitation of the computational sectioning).

To apply this method, certain requirements must be met regarding cutoff spatial frequency and intensity. Regarding cutoff spatial frequency, the algorithm utilizes a low-pass filter with a single cutoff frequency, which can make it challenging to accurately extract structures in the focal plane when structures of varying sizes and shapes are mixed within the sample. This is illustrated by the simulation shown in Fig. S5 and described in Note 2. Conversely, regarding intensity, if the structure's intensity in the focal plane is weak compared to the Gaussian fluctuations in the background intensity, it becomes difficult to extract the structure. However, intensity fluctuations can be reduced by applying a 3x3 moving average filter to the entire image as a pre-processing step before applying the baseline estimation algorithm.

In the experimental data presented in this paper (Figs. 4-6 in the revised manuscript), the spatial frequency issue was not significant because the target structures, which are stained nuclei, appear to be of nearly uniform size in the focal plane. The second issue, related to intensity, is also addressed in Fig. 4, as the signal intensity from the focal plane is sufficient to overcome background light in almost all regions. In the mouse brain example, the use of confocal imaging suppresses background light, allowing the structures in the focal plane to be accurately extracted.

(3) I didn't see a detailed description of the confocal imaging in the manuscript. If it adheres to conventional confocal technology, then the question arises: what truly constitutes the novel aspect of this technique?

The principle of confocal imaging and optics is based on the use of a pinhole array, a system also employed commercially by CrestOptics (X-Light, Italy). Prior to the 1990s, when the configuration utilizing Yokogawa Electric's pinhole array and microlens array pairs became

popular, pinhole array-only setups were the norm, and are now considered somewhat traditional. We do not claim novelty in the optical configuration itself, but rather in the design of a confocal optical system tailored for our original large-field (low-magnification) imaging system with a relatively high NA. The pinhole array disk we designed features significantly smaller pinholes and correspondingly tighter pinhole spacing than those used for high-magnification observation purposes. We believe that this unique size and arrangement provides sufficient novelty.

We have revised the manuscript to clearly emphasize what we believe constitutes the novelty of this technique (paragraphs starting from Line 166 and Line 183). We have also added a discussion on our confocal optical configuration and its spatial resolution in the Supplementary File (Note 1, Fig. S2-3).

(4) Light-sheet and light-field microscopy, as two emerging 3D microscopy techniques which has theoretically higher throughput than confocal, are not sufficiently introduced in this manuscript.

In the previous version, we briefly mentioned light-sheet and light-field microscopy, but we recognized that more detailed explanations were necessary and should be included in the manuscript. We have added several sentences to the main text (Line 159-165). A summary is provided below.

Light-sheet microscopy requires the illumination light to propagate over long distances within the specimen, and many applications necessitate the use of transparency-enhanced tissue. Even when the sample is highly transparent, no existing technique can form thin optical sections as long as 1 cm. Therefore, light-sheet microscopy is not an effective method for the thin, wide, three-dimensional objects that are the focus of this project. Regarding light-field microscopy, it features a trade-off where the number of pixels in the two-dimensional plane is reduced in exchange for the ability to record three-dimensional fluorescence distribution information in a single shot. In our imaging system, the pixel spacing is set to be comparable to the Nyquist Frequency to observe as many cells as possible, meaning that no more additional pixels can be sacrificed. Therefore, the light-field microscopy technique is not suitable for our imaging system.

(5) The fluorescence images of cardiomyocytes derived from human induced pluripotent stem cells (hiPSCs) stained with Rhodamine phalloidin, as presented in Figure 1(E), exhibit suboptimal quality. This may hinder the effective use of the image for biological research. It is imperative that the authors address and explain this aspect, shedding light on the limitations and potential implications of the research findings.

We acknowledge the reviewer's concern regarding the suboptimal quality of the fluorescence image. Upon further examination, we recognized that the resolution and clarity of the image could potentially limit its utility for detailed biological analysis. To address this, we have re-examined the image size and quality to enhance its presentation in Fig. 2C-E in the revised manuscript, which allows for finer structures to be recognized within the large image size.

Regarding the effective use of the image for biological research, the results shown in the images indicated the capability of observing subcellular structures, such as myofibrils, in cell sheets with a large area, such as myocardial sheets. This would enable us to simultaneously investigate micro-level structures (orientation and density of myofibrils) and macro-level multicellular dynamics (performance of myocardial sheet). We added the above explanation in the manuscript (Line 146). We hope this revision clarifies the quality and utility of the presented image.

(6) The imaging quality difference between the two techniques shown in Figure 1F, G is relatively small, and the signal distribution difference shown in Figure H is significant, unlike the effects expected from an improvement in resolution.

We acknowledge the reviewer's concern regarding the minimal apparent difference in imaging quality between the two images. Upon re-evaluation, we recognized that the original presentation may not have clearly demonstrated the improvements intended by the different techniques. Figure 1H, which showed the line profile of Figs. 1F and G, may have been impacted by the resolution and compression settings of the image file, leading to a less pronounced distinction between the two techniques. To address this, we have enlarged Figs 1F and 1G

(renumbered as Fig. 2D and 2E in the revised manuscript) and carefully reviewed the resolution and compression ratio to ensure that the differences are more clearly visible.

(7) The chart in Figure 2(C) lacks axis titles and numerical labels, making it challenging for readers to comprehend. To enhance reader convenience, it is recommended that the authors incorporate axis titles and numerical labels, providing a clearer context for interpreting the chart.

We appreciate the reviewer's observation regarding the lack of axis titles and numerical labels in the figure. The vertical axis represents fluorescence intensity, which we initially omitted, assuming it was self-evident. However, as the reviewer correctly pointed out, it is crucial to ensure that figures are clear and accessible to readers from diverse backgrounds. In response, we have added the vertical axis title to Fig. 2C (renumbered as Fig. 3C in the revised manuscript) to enhance clarity, while the numerical labels remain omitted as the unit is arbitrary (a.u.). We have also reviewed all other figures in the manuscript to ensure that no similar errors are present.

(8) In Figures 2(D) and (E), where the authors present the point spread function for quantifying the lateral and axial resolution of the system, I would recommend increasing the number of fluorescent microspheres to more than 10 for statistical averaging. This adjustment would strengthen the persuasiveness of the data and contribute to a more robust analysis.

We appreciate the reviewer's recommendation to increase the number of fluorescent microspheres for statistical averaging in Figs. 2D and E (renumbered as Fig. 3D-E in the revised manuscript). In response, we have revised the graphs to present the point spread function with the statistical mean and standard deviation (SD) of fluorescent images obtained from a large sample size ($N = 100$), and accordingly revised the main text to mention the statistics (Line 118, Line 132). We also recognized that a similar adjustment was necessary for Figs 1C and D (renumbered as Fig. 2A-B in the revised manuscript), and have accordingly made the same modifications to those figures as well. We believe these changes enhance the robustness and persuasiveness of our data.

(9) Figure 4(C) visually represents the characteristic 3D structures of several regions. However, discerning the 3D structural information in the images poses a challenge. To address this issue, I recommend that the authors optimize the 3D visualization to improve clarity and facilitate a more effective interpretation of the depicted structures.

We appreciate the reviewer's suggestion regarding the challenges in discerning the 3D structural information in Fig. 4C. To address this, we have added representative images from the xy-plane and xz-plane of the cortex, medial habenula, and choroid plexus (Fig. 5G-I) in the

revised manuscript. These additions provide a clearer visualization of the 3D distribution in each region, making it easier for readers to interpret the structures. Additionally, we have overlaid the results of deep-learning based cell detection on these images, further enhancing the visibility of the cells. This adjustment also aligns with our response to Reviewer #1's second comment.

Minor comments:

(1) The labelling of ROI is missing in Figure 1(e).

We appreciate the reviewer's observation regarding the missing labeling of the ROI in Fig. 1E. Upon review, we confirmed that the ROI was indeed labeled with a white square in the previous manuscript; however, it was difficult to discern due to its small size and the black-and-white contrast. To improve visibility, we have recolored the square in magenta, ensuring that it stands out more clearly in the figure (Fig. 2C in the revised manuscript).

(2) The subfigure order and labeling in Fig. 1 and Fig. 2 are not consistent.

We appreciate the reviewer's attention to the subfigure order and labeling in Fig. 1 and 2 (Fig. 1-3 in the revised manuscript). To accommodate subfigures of varying sizes without leaving gaps, we arranged the subfigures in a non-sequential order. However, we have ensured that the text refers to the figures in the correct order. We acknowledge the importance of consistency and will work with the editorial team to explore the best way to present the figures while maintaining clarity and alignment with standard practices.

(3) Figure 1B reappears in Figure 2.

We appreciate the reviewer's observation regarding the repetition of Figure 1B in Figure 2. While the central part of the optical system (custom lens system) is common to both figures, the illumination system, pinhole array disk, and detection optics for the confocal set up differ. To provide a complete understanding of the optical system, we opted to include the full diagram in Fig. 2B (renumbered as Fig. 3B in the revised manuscript). We considered highlighting only the different components, but we felt that doing so might complicate the reader's comprehension of the overall system. Therefore, we chose to include the common elements twice to ensure clarity.

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