Abstract

Fluorescence imaging is indispensable to biology and neuroscience. The need for large-scale imaging in freely behaving animals has further driven the development in miniaturized microscopes (miniscopes). However, conventional microscopes / miniscopes are inherently constrained by their limited space-bandwidth-product, shallow depth-of-field, and the inability to resolve 3D distributed emitters. Here, we present a Computational Miniature Mesoscope (CM₂) that overcomes these bottlenecks and enables single-shot 3D imaging across an 8 × 7-mm² field-of-view and 2.5-mm depth-of-field, achieving 7-µm lateral and 250-µm axial resolution. Notably, the CM₂ has a compact lightweight design that integrates a microlens array for imaging and an LED array for excitation in a single platform. Its expanded imaging capability is enabled by computational imaging that augments the optics by algorithms. We experimentally validate the mesoscopic 3D imaging capability on volumetrically distributed fluorescent beads and fibers. We further quantify the effects of bulk scattering and background fluorescence on phantom experiments.
Introduction

Fluorescence microscopy is an indispensable tool in fundamental biology and systems neuroscience (1). A major focus for recent technological developments is aimed at overcoming the barrier of scale (2). For example, perception and cognition arise from extended brain networks spanning millimeters to centimeters (3), yet rely on computations performed by individual neurons only a few microns in size (4). Recent progress, such as macrosopes (3), Mesolens microscope (5), two-photon mesoscope (6), and RUSH (7), are only beginning to bridge these scales. However, the development of such mesoscopic imaging systems is confounded by the scale-dependent geometric aberrations of optical elements (8). This results in an undesirable tradeoff between the achievable space-bandwidth-product (SBP) and the complexity of the optical design (8, 9), as evident by mesoscopes developed based on both the sequential (5, 6) and multiscale (7) lens design principles. In addition, the achievable field-of-view (FOV) is further constrained by the system’s shallow depth-of-field (DOF) in many bioimaging applications (3, 7). For example, the FOVs for cortex-wide imaging systems are often set by the curved cortical surface that requires additional mechanisms to be compensated for, otherwise resulting in excessive out-of-focus blurs in the peripheral FOV regions (3).

Another technological focus is towards miniaturization driven by the need of long-term in vivo imaging in freely behaving animals. In particular, miniaturized head-mounted fluorescence microscopes, i.e. “miniscopes” (10), have made significant progress and enabled unprecedented access to neural signals, revealing new views of neural circuits underlying diverse behaviors such as navigation, memory storage, learned motor programs, and social interactions. However, the imaging performance of current miniscope systems remains restricted by their optics, similar to their standard fluorescence microscopy counterparts. Most importantly, multiscale measurements are still beyond reach. Most of current miniscope systems limit imaging areas to under 1 mm² (10), confining measurements to a subset of cells within a single brain region. While larger FOVs are possible, fundamental physical limits preclude meeting the joint requirements of scale, resolution, and compactness by simply scaling up standard optical designs (11). In addition, widefield measurements only give access to fluorescence signals within a limited depth of several micrometers around the plane of focus, as set by the DOF of the optics (10). The head-mounted configuration further constrains the flexibility of adjusting focus, making imaging of 3D distributed emitters highly challenging (12). Although two-photon miniscopes have been developed to provide 3D scanning capability (13, 14), they require specialized optics and suffer from slow acquisition speed (10).

Here, we introduce and demonstrate a Computational Miniature Mesoscope (CM²) that enables large-scale 3D fluorescence measurements with a compact and lightweight optical platform. The CM² uses simple optics and accomplishes its SBP improvement and 3D imaging capability without the need for mechanical scanning. It bypasses the physical limitations of the optics by jointly designing the hardware and algorithm. Specifically, the CM² is capable of reconstructing 3D fluorescence distributions in 8.1 × 7.3 × 2.5 mm³ volumes and achieving ~7 μm lateral and ~250 μm axial resolution from a single widefield measurement. This represents at least one order of magnitude increase in the FOV and two orders of magnitude improvement in the DOF over current miniscope systems, while still offering cellular level resolution.
The imaging method of CM² combines ideas from integral imaging (15), lightfield microscopy (16), compound-eye imaging (17), array microscopy (18, 19), and coded aperture imaging (20–23). It works by first collecting a single 2D measurement using a microlens array (MLA), and then computationally reconstructing the 3D fluorescence distribution based on pre-characterized PSFs. Unlike systems designed to acquire 3D information by attaching an MLA to an existing microscope (12, 16, 24), the CM² uses the MLA as the sole imaging element (Figs. 1A and 1B), allowing our setup to circumvent the FOV limitations imposed by conventional objective lenses. In addition, this configuration offers a simple and compact form factor by removing the bulk of infinite-conjugate optics used in existing miniscopes (10, 12). Similar to coded-aperture techniques (20–23), CM² captures 3D information through optical multiplexing, where the PSF is no longer a single tight focus but spreads over multiple foci (Fig. 2A). As compared to techniques using highly dispersed PSFs (20, 21), CM² is designed to have a “small” 3 × 3 focal spot array that incurs a proportionally low degree of multiplexing, since both contrast and signal-to-noise ratio (SNR) degrade as multiplexing increases (25). This design ensures CM² captures high-contrast measurements containing 9 partially overlapping perspective projections from the object (see Figs. 1C and 2D). Furthermore, the multi-view finite-conjugate configuration provides CM² with rapidly varying point spread functions (PSFs) across depths (Fig. 2A), which lays the foundation for robust and accurate 3D reconstruction. Accordingly, the forward model of the CM² describes the convolution between the 3D object and the depth-dependent PSFs simultaneously projected onto the image sensor. The CM² reconstruction algorithm recovers the 3D object by solving a sparsity-promoting regularized least-squares problem. As compared to the digital refocusing algorithm that synthesizes geometrically refocused images at different depths (15, 16, 19), the CM² algorithm provides depth-resolved reconstructions by solving the full shift-variant 3D-to-2D deconvolution problem.

Importantly, CM² operates as a standalone fluorescence imaging device that integrates the fluorescence excitation module with the imaging module on the same platform (Figs. 1A and 1B). Naïvely adopting the popular on-axis epi-illumination to a mesoscale FOV leads to bulky optics and undesired long working distance. Instead, we design and optimize an array of LEDs that create uniformly distributed illumination across a centimeter-scale FOV using an oblique epi-illumination configuration. In addition, this design imparts the compactness and light weight of the CM² and bypasses the conventional limitations from the collimating optics, dichromatic mirror (10), and diffusing elements needed for wide-FOV illumination (11) in existing miniscopes.

Building from off-the-shelf hardware components, a 3D printed housing, and augmented with the reconstruction algorithm, the CM² enables volumetric imaging of fluorescence objects and provides high-resolution, wide-FOV, and extended DOF with a final SBP of approximately 20.7 million voxels. Our joint optical-computational design allows us to perform tomographic reconstruction using a single measurement. In the following, we first briefly outline the operation principle of the CM² and derive the theoretically achievable lateral and axial resolution based on a 3D modulation transfer function (MTF) analysis. We then show that our experimentally obtained resolution matches well with the theoretical predictions. Next, we experimentally demonstrate the 3D mesoscopy capability under different imaging conditions. First, we present results on scattering-free samples, including fluorescent particles embedded in clear volumes and fiber clusters spread over a curved surface. Next, we investigate the effects of bulk scattering and background fluorescence, and then quantify the axial reconstruction range of the CM² in a series of controlled phantom
experiments. Finally, we demonstrate the advantage of the CM²’s extended DOF across a mesoscale FOV by imaging a scattering volume with a curved surface geometry.

Results

**Principle of the CM²**

The principle of the CM²’s single-shot 3D imaging capability can be explained by drawing an analogy to the frequency division multiplexing (FDM). In the FDM, simultaneously transmitting multiple signals is made possible by first modulating each signal with a distinct carrier signal and later separating them by demodulation. Analogously, in the CM² simultaneously resolving fluorescence signals from multiple depths is achieved by first convolving the signals from each depth with a distinct PSF and later reconstructing the depth-wise information by deconvolution. Further considering fluorescence signals from a continuous volume, the axial resolving power of the CM² is fundamentally limited by the need for substantially differing PSFs across depths. This can be quantified using the correlation-coefficient calculated between the nominal in-focus PSF ($z = 0$) and each out-of-focus PSF. As shown in Fig. 2A using the experimentally measured PSFs, the correlation reduces rapidly across different depth planes because of the axial sheering of the array foci. We further compared our $3 \times 3$ MLA design with other configurations, including a single microlens and a $2 \times 2$ MLA, and show the decorrelation improves with the number of microlenses used. Recall that increasing the number of microlenses, however, reduces the image contrast and the SNR. Our design seeks the balance between the three imaging attributes while minimizing the size and weight.

The achievable resolution of the CM² is further quantified by computing the system’s 3D MTF. In Fig. 2B, the XZ cross-sections of the 3D MTFs of the CM² and the single-microlens system are compared (see details in Materials and Methods). Notably, the CM² dramatically extends the axial ($f_z$) bandwidth, and hence achieves much improved axial resolution. Akin to all microscopes (26), the axial bandwidth of the CM² is dependent on the lateral frequency ($f_x$). High-resolution features (e.g. feature size $< 10 \, \mu$m corresponding to $f_x > 0.1 \, \mu$m$^{-1}$) can be resolved axially with high resolution at $\sim 230 \, \mu$m. The low frequency region suffers from the common “missing-cone” problem (26), leading to deteriorated axial sectioning. The lateral ($f_x$) bandwidth of CM² is $\sim 0.2 \, \mu$m$^{-1}$, corresponding to $\sim 5 \, \mu$m lateral resolution. The lateral bandwidth is not strongly affected by axial frequency, indicating a relatively uniform lateral resolution regardless of the axial feature size. As compared to the single-microlens case, the CM² does not expand the lateral bandwidth, however the modulation contrast is much improved at high lateral frequency regions. This means that these high-resolution features are more tolerant to noise contaminations and can be more faithfully reconstructed because of the improved conditioning of the underlying inverse problem (27). We later experimentally verify these theoretical predictions on fluorescent samples with different feature sizes and demonstrate excellent match between the theory and the experiments.

**Lateral resolution test**

A fluorescent resolution target (Edmund Optics 57-895) is imaged to determine the lateral resolution of the CM². We conducted experiments by tilting the target across the volume (Figs. 2C-E) or placing it on the same focal plane (Fig. S4A) and show that the same lateral resolution is achieved regardless of the geometry. The XY maximum intensity projection of the CM² reconstruction (Fig. 2E) shows that the features separated by $\sim 7 \, \mu$m (Group 6, Element 2) can be resolved, matching our MTF analysis. The XZ maximum intensity
projection (Fig. 2E) demonstrates successful recovery of the tilted geometry. The feature size-dependent axial sectioning is also observed. The larger features result in wider axial elongations. Since we took the measurement by placing the high-resolution features in the central region of the CM²’s FOV, the axial elongation artifacts are observed more prominently in the outer FOV regions corresponding to the low-resolution features on the target. We further validated these observations using Zemax simulated measurements (Supplementary Materials Fig. S4B and Section S5) and find good agreement between the simulations and the experiments.

**Experiments on fluorescent particles in clear volumes**

We experimentally demonstrate that the CM² allows localizing fluorescent emitters distributed across a large volume. First, we imaged 100-μm diameter fluorescent particles dispersed in a ~7.0 mm × 7.3 mm × 2.6 mm volume (the CM² measurement shown in Fig. 1C). We establish the accuracy of the CM² volumetric reconstruction result (Fig. 1D and Supplementary Materials Movie S1) by comparing it with an axial stack acquired using a 10×0.25 NA objective on a commercial epi-fluorescence microscope (Nikon TE2000-U) in Supplementary Materials Fig. S7B and Section S9 and show excellent agreement between the two. The axial elongation from each 100-μm particle is consistently around 420 μm, which matches with our 3D MTF analysis predicting 476-μm axial resolution (axial cutoff frequency = 0.0021 μm⁻¹) at \( f_x = 0.01 \) μm⁻¹. The slight improvement is possibly from the sparsity-promoting reconstruction algorithm.

Next, to test the CM²’s performance on samples with sizes similar to neurons, we imaged 10-µm diameter fluorescent particles distributed in a ~5.7 mm × 6.0 mm × 1.0 mm volume. The raw CM² measurement is contaminated with stronger background fluorescence due to the increased particle concentration and suffers from lower contrast and reduced SNR due to reduced brightness of the emitters (shown in the insert in Fig. 3A). Nevertheless, the CM² algorithm is tolerant to these signal degradations, as shown in the high-quality full-FOV reconstruction in Fig. 3A. The mesoscale FOV offered by the CM² is highlighted by comparing that from the 2× and 10× objective lenses (on Nikon TE2000-U microscope with a PCO Edge 5.5 sCMOS). Visual comparison between the CM² reconstruction and the axial stack acquired by the 10×0.25 NA objective are shown in Fig. 3B, demonstrating accurate single-shot localization of each particles (volumetric visualization available in Supplementary Materials Movie S2). We further quantify the reconstruction accuracy by comparing the CM² reconstruction with the axial stacks taken with 2×0.1 NA and 10×0.25 NA objective lenses. As shown in the lateral cross-sections in Fig. 3C, the CM² accurately recovers the 10-µm particle profile. Further evaluating the axial cross-sections (Fig. 3C) indicates that the CM² reconstruction achieves better axial sectioning than the 0.1 NA objective lens but worse than the 0.25 NA objective lens. The XZ cross-sectional view of a single-particle reconstruction (Fig. 3D) also highlights these observations. Quantitatively, the axial elongation from each 10-μm particle is consistently around 250 μm, matching the 3D MTF predicted 232 μm axial resolution (axial cutoff frequency = 0.0043 μm⁻¹) at \( f_x = 0.1 \) μm⁻¹.

**Experiments on fluorescent fibers on a curved surface**

The ability to image complex volumetric fluorescent samples is experimentally tested on fluorescent fibers spread on a 3D printed curved surface that mimics the surface profile of a mouse cortex (as shown in Fig. 4A). The sample spans a wide FOV (~7.8 mm × 4.9 mm) and an extended depth (~0.9 mm). As shown in the depth-color coded projections of the full-FOV reconstruction (Fig. 4B and Supplementary Materials Movie S3), the overall
As the scattering increases, the reconstruction quality of the CM² reconstruction is highlighted by comparing a few reconstructed depths with the widefield fluorescence measurements using the 2× 0.1 NA and 10× 0.25 NA objective lenses (Fig. 4D). The CM² algorithm correctly recovers the in-focus structures and rejects the out-of-focus blurs in each depth, since it solves for the 3D object rather than mimicking the physical focusing on a microscope. We also plot the reconstruction cutline across a dense fiber cluster and compare it with the widefield measurement from the 2× 0.1 NA objective lens. The overlay verifies that the CM² resolves most of the individual fibers (Fig. 4C). The difference in the intensity of different fibers between the two cutlines are primarily due to the different illumination conditions used during the measurements (oblique epi-illumination for the CM² vs on-axis for the 2× objective lens on a standard epi-fluorescence microscope). Additional experiments on the same type of fluorescent fibers placed on a planar surface are conducted to further verify the above observations, as described in Supplementary Materials Section S8 and Fig. S7A.

**Experiments on controlled scattering phantoms**

To quantitatively evaluate the performance of the CM² under bulk scattering and strong background fluorescence, we conducted experiments on eight phantoms with progressively increasing scattering properties, as summarized in Fig. 5. All the phantoms are seeded with the same concentrations of the background fluorescent particles (1.1-µm fluorescent beads) and the target fluorescent particles (25 µm fluorescent beads). The first phantom does not contain any additional scatterers. For the rest of the seven phantoms, we doubled the seeding density of the non-fluorescent scatterers (1-µm polystyrene beads) in each sample (see more details in Materials and Methods).

The raw CM² measurement is subject to strong background and reduced image contrast, as shown in the example image (the top left panel of Fig. 5A). To overcome this issue, we conducted a background subtraction procedure (as detailed in Materials and Methods) before performing the 3D deconvolution. This procedure can effectively remove the slowly varying background while maintaining high-fidelity signals from the high-contrast targets, as shown in the background removed image (the top right panel of Fig. 5A) and the overlay between the raw and background removed images (the bottom panel of Fig. 5A). To quantify the effects of background fluorescence and bulk scattering in the raw measurements, we calculated the signal-to-background ratio (SBR) (see details in Materials and Methods) and found that the SBR is reduced from ~1.62 to ~1.18 in our experiments (the blue curve in Fig. 5B).

We performed 3D reconstruction for each scattering phantom. In order to reach a fair and comparison between the results, all the deconvolutions are conducted using the same computational settings (i.e. the same set of regularization parameters and a fixed number of iterations), so that the influence from the (nonlinear) regularization terms can be considered approximately identical across all cases. The reconstruction results are visualized in the XZ maximum intensity projections in Fig. 5C. As the scattering increases, while it is still possible to resolve individual emitters, the reconstructed depth range gradually reduces. When the scattering is sufficiently strong, the CM² reconstructs the emitters within the superficial layer of the phantom (Phantoms 6-8). This observation is further quantified by measuring the reconstructed depth range in each case (the red curve in Fig. 5B) (more details in Materials and Methods). It is generally observed that the depth range reduces as the SBR reduces. The depth range is first limited by the background fluorescence (Phantoms 1-5). As the scattering increases, the range approaches the limit set by the single scattering mean

Surface curvature with closely packed fiber structures can be clearly recovered. The reconstruction quality of the CM² reconstruction is highlighted by comparing a few reconstructed depths with the widefield fluorescence measurements using the 2× 0.1 NA and 10× 0.25 NA objective lenses (Fig. 4D). The CM² algorithm correctly recovers the in-focus structures and rejects the out-of-focus blurs in each depth, since it solves for the 3D object rather than mimicking the physical focusing on a microscope. We also plot the reconstruction cutline across a dense fiber cluster and compare it with the widefield measurement from the 2× 0.1 NA objective lens. The overlay verifies that the CM² resolves most of the individual fibers (Fig. 4C). The difference in the intensity of different fibers between the two cutlines are primarily due to the different illumination conditions used during the measurements (oblique epi-illumination for the CM² vs on-axis for the 2× objective lens on a standard epi-fluorescence microscope). Additional experiments on the same type of fluorescent fibers placed on a planar surface are conducted to further verify the above observations, as described in Supplementary Materials Section S8 and Fig. S7A.

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free path (Phantoms 6-8), much like other widefield fluorescence techniques. When the scattering mean free path is shorter than the axial elongation from the 25-µm fluorescence particle (~370 µm), the experimentally measured depth range in Fig. 5B is set by this elongation due to limited axial resolution (Phantoms 7-8). The estimation of the reconstruction depth range is affected by the surface variations present in each phantom, as seen in the visualization in Fig. 5C, where the white dashed line represents the estimated top surface of each phantom in the reconstruction.

**Experiments on scattering sample with a curved surface geometry**

Although the reconstruction volume of the CM² is fundamentally limited by bulk scattering, next we show that it is still an effective solution of compensating for the surface curvature often present in a mesoscale FOV. To demonstrate this, we imaged a scattering (l ~ 264 µm) phantom with a curved surface geometry (Fig. 6A). The phantom was made using the same protocol as before. The entire surface spans approximately a 725-µm range. Although only the fluorescent emitters within the superficial layer can be recovered, the curvature of the surface is faithfully reconstructed by the CM², as highlighted in the reconstructed volume in Fig. 6B and visualized in Supplementary Materials Movie S4. The fidelity of the reconstruction is further validated against the widefield fluorescence measurements in Fig. 6C, which shows excellent agreement.

**Discussion**

In summary, a novel miniaturized fluorescence imaging system is demonstrated to enable single-shot mesoscopic 3D imaging. This compact and lightweight device integrates fluorescence imaging and excitation modules on the same platform. Simulations and experiments have been presented to establish the imaging principle and 3D imaging capability of the CM². Its utility for 3D mesoscopic imaging under bulk scattering and strong background fluorescence has been experimentally quantitatively evaluated. This computational microscopy technique achieves a cm²-scale FOV, a mm-scale DOF, ~7 µm lateral resolution, and ~250 µm axial resolution, which offers a large ~20.7 million-pixel information throughput in a single shot. Under bulk scattering, CM² is still able to reliably reconstruct the fluorescence distribution in the superficial layer and digitally compensate for curved surface geometries. With these unique combinations of imaging capabilities, we believe this novel system has a strong potential for achieving neural imaging on scales approaching the full extent of the mouse cortex while maintaining high resolution and light weight that is critical for chronic implants in mice. As a pilot study, we simulated a brain-wide imaging of vascular networks in Supplementary Materials Section S6 and Fig. S5. The results show promising results of imaging complex structures across a cortex-wide FOV and accommodating for mm-scale surface variations.

The imaging capability of the CM² can be further improved with future development in both hardware and algorithm. In the hardware, this first-generation device suffers from relatively low light efficiency (~25% overall efficiency) due to the oblique epi-geometry, limiting its application to weak fluorescent samples. A pilot study on imaging a Green fluorescent protein (GPF)-labeled mouse brain slice is described in Supplementary Materials Fig. S6 and Section S7, which demonstrates the mesoscopic imaging capability and the limitation of the current version of the CM². The light efficiency may be improved by alternative designs, such as using novel focusing optics (28), diffractive optical elements (29) or fiber optics-coupled light sources (30). The imaging capabilities (e.g. resolution, FOV, and DOF) may further be improved by using customized aperiodic microlens arrays (23, 31). On the
algorithm side, the reconstruction quality of the CM² is currently limited by its simplified depth-wise shift invariant model that ignores the slowly varying components in the PSFs in each lateral plane. The error induced by this approximation currently limits the practically achievable FOV, which is analyzed in more details in Supplementary Materials Fig. S10 and Section S12. This is coupled with the computational cost of the reconstruction algorithm that currently uses an iterative procedure. Both limitations may be alleviated by more advanced computational methods, such as the local convolution model (23), deep learning (32–34) and those incorporating temporal dynamics (35). With these improvements, we envision that future generations of the CM² may open up new exciting opportunities in a wide range of large-scale in vivo 3D neural recording and biomedical applications.
Materials and Methods

The CM² prototype
The CM² consists of two main parts for fluorescence imaging, including the imaging and illumination modules, as shown in Figs. 1A, and 1B. The detail descriptions are provided in the Supplementary Materials Section S1, and Fig. S1. Briefly, for the imaging path, we choose an off-the-shelf MLA (#630, Fresnel Technologies Inc., focal length = 3.3 mm, pitch = 1 mm, thickness = 3.3 mm). The MLA is first diced into a smaller array whose size is slightly larger than the 3 × 3 array (see Supplementary Materials Section S2). The extra size is needed to minimize vignetting due to the thickness of the MLA, as illustrated in the ray tracing (in Zemax) in Fig. S2. The 3 × 3 MLA is approximately centered about the CMOS image sensor (MT9P031, sensor area 4.3 mm × 5.7 mm) by a 3D printed housing. No precise alignment is needed. After assembly, a one-time system calibration procedure is performed, in which a point source is scanned along the optical axis of the MLA to acquire a stack of PSFs (see Section PSF calibration and Supplementary Materials Section S3). A thin emission filter (535/50, Chroma Technology) is placed between the MLA and the sensor.

For the illumination path, four surface mounted LEDs (LXML-PB01-0040, Lumileds) are placed symmetrically around the MLA for fluorescence excitation to provide oblique epi-illumination. The LED is connected to a driver (350 mA, 3021-D-E-350, LEDdynamics Inc.). Each LED is first filtered spectrally by the excitation filter (470/40, Chroma Technology), and then angularly confined by a 3D printed aperture to generate an oblique diverging beam for illuminating the imaging region. The positioning of the LEDs and the 3D printed apertures are optimized. To do so, we build a model in Zemax that incorporates the array geometry, the LED spectrum, the angular profile of each LED emitter, and the incident angle-dependent transmittance profiles of the excitation filer. We then optimize a merit function that considers the illumination uniformity and the total flux. Additional details about the illumination optimization is given in Supplementary Material Section S10. We achieve highly uniform illumination over a 10 mm × 10 mm area after the optimization, as shown in Fig. S8. The oblique geometry also reduces the transmittance efficiency of the emission filter, which is also modeled by our Zemax model (see Supplementary Material Section S11 and Fig. S9).

The prototype is built around the FLIR BFLY-PGE-50A2M-CS platform by modifying the original aluminum housing with 3D printed parts using black resin materials (Form 2, FormLabs). Additionally, linear polarizing thin films (86-180, Edmund Optics) are inserted in front of each LED and the MLA. The orientation of each polarizer is adjusted to achieve optimal rejection of the specular reflections. After assembly, the prototype is connected to a desktop computer via an Ethernet cable for power and image acquisition.

PSF calibration
After the CM² is assembled, it only requires a one-time calibration to characterize its PSFs. To perform the calibration, we first build a point source consisting of a green surface-mounted LED (M530L4, Thorlabs), diffused by multiple layers of highly scattering thin films (Parafilm), and followed by a 5-µm pinhole (P5D, Thorlabs). Details on the construction of the point source are in the Supplementary Material Section S3 and Fig. S3. The point source is mounted on a three-axis automatic translation stage and controlled by a custom-built MATLAB program. To calibrate the PSFs, the point source is scanned along the axial direction with a 10-µm step size across the [-3.5 mm - 3.5 mm] range. The
measured PSFs are later registered numerically to account for the slight misalignment between the mechanical scanning axis and the optical axis.

### 3D MTF calculation

After acquiring the system’s PSF stack, we estimate the theoretically achievable resolution by computing the system’s 3D MTF. Note that the MTF calculation assumes the system to be spatially shift invariant. However, CM² is a shift variant system due to the finite-sized image sensor that may truncates the PSFs and the spatial varying aberrations. By ignoring these shift variance effects, the 3D MTF is calculated by directly taking the 3D Fourier transform on the PSF stack.

### The CM² forward model and reconstruction algorithm

The CM² measurement can be modeled as the sum of the object at each depth convolved with each depth-dependent PSF. It further assumes an unknown boundary condition by including a truncation operation (36) to account for the limited image sensor size. At a fixed depth, the PSF also changes slowly across the FOV. However, to fully account for both lateral and axial shift variance incurs large costs from the physical PSF calibration and computation. We simplify the model by neglecting the lateral shift variance. The degree of lateral shift variance is characterized in Supplementary Materials Section S1.

This simplification leads to a reduced FOV that can be reconstructed due to the model mismatch in the peripheral imaging area (as shown in Fig. S10). Concretely, the CM² forward model can be written as the following compact form,

\[
y = DHx,
\]

where the discretized 3D object \(x = [x_1, x_2, \ldots, x_n]^T\) is written as \(n\) discrete depth slices that are concatenated into a long vector. The convolution operator \(H = [H_1, H_2, \ldots, H_n]\) stacks all the corresponding 2D convolution matrix. The operation \(Hx = \sum_{i=1}^{n} H_i x_i\) effectively projects all the 2D measurement \((H_i x_i)\) from different depths onto the same image sensor. \(D\) further accounts for the truncation due to the finite sized image sensor.

The reconstruction algorithm solves an inverse problem that is highly ill-posed because of the dimensionality mismatch (i.e. from 2D to 3D). Our strategy is to incorporate priors by solving the following constrained optimization

\[
\hat{x} = \arg\min_{x \geq 0} \frac{1}{2} \|DHx - y\|_2^2 + R(x)
\]

where \(R\) includes two types of regularization terms, including the \(l_1\)-norm the and 3D total variation. The non-negativity constraint enforces the recovered fluorescent intensity to be positive and is achieved by minimizing an indicator function \(1_+ (\cdot)\). To efficiently solve this regularized least-squares problem, we adopt the alternating direction method of multipliers (ADMM) algorithm (37) by splitting the problem into a sequence of sub-optimizations, where each sub-optimization has either a closed form solution or a fast proximal operator (36). The iterative algorithm typically takes 0.5 and 2.5 hours to converge for 2D planar and 3D volumetric objects respectively. The reconstruction algorithm is implemented in MATLAB 2018b and runs on the Boston University Shared Computing Cluster with an Intel Xeon Processor E5-2650 v2. The typical memory requirement is 16GB and 256GB for planar and volumetric object reconstructions, respectively. Additional details of the algorithm are provided in Supplementary Materials Section S4.
Background subtraction algorithm
We perform background subtraction on the raw CM² measurement to remove the slowly varying background before performing the 3D deconvolution, implemented in MATLAB 2018b. The background is estimated by applying the image morphology opening algorithm to the raw image, which is a two-step morphological operation. It first performs an image erosion followed by a dilation, both with the same template. In our case, the template is chosen to be a disk with the diameter greater than the size of the fluorescent targets. It is observed that in the scattering phantom experiments, the signals from the emitters below the superficial layer are removed by this background subtraction procedure since these signals generally have low contrast and spread out over a much larger area compared to those at the superficial layer.

Quantification of the signal-to-background ratio (SBR)
For the experiments on phantoms with different scattering densities. We quantify the signal-to-background ratio (SBR) of the raw CM² measurements for each phantom. The SBR is calculated as the mean intensity value on particle region over the mean intensity value on the background region. In our typical measurement, the background is not uniform across the whole FOV. To account for the background variations, we randomly select multiple sub-FOVs (500 μm × 500 μm) from the raw measurements and quantify the local SBRs on each selected image patch. When collecting sub-FOV patches, we remove the regions that contain bead clusters or boundary artifacts, such as glare to avoid abnormal SBR values. The mean and standard deviations of the calculated SBRs from each phantom are plotted in Fig. 5B. As expected, the SBR reduces as the scattering density increases. This also matches with the visual inspections of the raw measurements shown in Fig. 5C.

Quantification of the reconstructed depth range
To quantify the imaging depth limit of the CM² under bulk scattering, we measure the reconstructed depth range for each phantom. This is done by first randomly selecting multiple sub-FOV patches (800 μm × 800 μm) from the reconstructed volumes and then taking the XZ maximum intensity projections. We then use the 3D object counter tool in ImageJ to detect the centroid of each reconstructed particle. For each sub-FOV, the local depth range is calculated as the difference between the maximum and minimum axial coordinates of the centroids with the additional axial elongation from each particle (372 μm at $f_s = 0.04 \text{ μm}^{-1}$) to account for the intrinsic uncertainty in this measurement determined by the finite axial resolution. Lastly, the mean and standard deviation are calculated from multiple selected sub-FOVs, as reported in Fig. 5B. It is observed that the recovered depth range reduces as the SBR decreases. Additionally, the depth range curve crosses the scattering mean free path curve at Phantom 6 ($l_s = 497 \text{ μm}$) and later plateaus. This is because when the scattering mean free path (497 μm) is close to the axial resolution (372 μm), only emitters at the superficial layer of the sample can be faithfully reconstructed. When the scattering mean free path is less than the axial resolution, the reconstructed depth range is set by the blur induced by the imaging optics.

Zemax simulation
We conducted two series of simulations in Zemax, including study of the imaging path and the illumination path of the CM². In the imaging path simulation, we used either a standard resolution target or a simulated mouse brain vasculature network as the sample. To make the simulation match the experimental conditions, the model incorporates the shift variant aberrations in the CM² by performing ray tracing in Zemax. All the components used in the model, including the MLA, image sensor, and the 3D printed housing, are modeled to match
the actual sizes in Zemax. The objects used in the simulation are first generated and then imported into the non-sequential mode of Zemax as source objects. In particular, the mouse brain vasculature object is generated by discretizing the volume into 16 discrete layers with a 0.1 mm layer thickness. Furthermore, to account for the filter efficiency change under oblique illumination, we imported the incidence dependent transmittance profiles of the filter set from the manufacturer (Fig. S9), as well as the emission and excitation spectra of the fluorophores used in the experiments. In the illumination path simulation, we optimize the uniformity and efficiency over ~1 cm² excitation area. The surface mounted LEDs, along with the 3D printed housing and filters, are accurately modeled in terms of their positions and spectral characteristics. A virtual detector is placed at the desired sample plane to measure the intensity of the excitation beam. Additional details can be found in Supplementary Materials Section S10 and Fig. S8.

**Calculation of the space-bandwidth product**

The SBP of a 3D imaging system (3D SBP) measures the fundamental information throughput which is equivalent to the maximum number of voxels that can be resolved inside the imaging volume. This is calculated by the ratio between the imaging volume and the size of the 3D PSF. Our experimentally measured maximum FOV and DOF are 8.1 mm × 7.3 mm and 2.5 mm, respectively. The experimentally obtained best lateral resolution is 6.9 μm (group 6, element 2 on the 1951 USAF resolution target). According to our 3D MTF analysis, the axial resolution of CM² depends on the lateral feature size. Therefore, the practically achievable 3D SBP depends on the frequency content of the actual object. To give a reasonable estimate of the SBP, we calculate the upper bound of the SBP based on the highest resolved spatial frequency ($f_x = 0.14 \mu m^{-1}$). From our experimentally computed 3D MTF, the axial cutoff frequency at $f_x = 0.14 \mu m^{-1}$ is ~0.0067 $\mu m^{-1}$, which corresponds to an axial resolution of ~150 μm. Therefore, the highest 3D SBP of the CM² is ~20.7 million.

**Imaging of the fluorescent resolution target**

The resolution target needs to be excited at 365 nm and emits at 550 nm, which does not match our choice of LEDs in the CM² (designed for exciting common Green Fluorescence Proteins). As a result, the measurements of the target were taken with an external UV lamp.

**Scattering-free sample preparation**

Fluorescent particles with different sizes (10-μm, 100-μm, Thermo Scientific Fluoro-Max Green Dry Fluorescent Particles) are first suspended in the clear resin (FormLabs, #RS-F2-GPCL-04) and then diluted to different concentrations. Next, we apply the mixture onto a standard 1-inch microscope slide. The samples are later cured under a UV lamp. The samples are controlled to be within 1~2 mm in thickness. The fluorescent fiber sample is made by soaking lens tissue fibers in green fluorescent dyes and then cured inside the clear resin. To mimic the surface curvature of mouse cortical cerebral, fluorescent fibers are first placed on top of a 3D printed clear mouse brain model to form a total depth range of around 1 mm.

**Scattering phantom preparation**

We fabricate scattering phantoms with both bulk scattering and background fluorescence. The bulk scattering is controlled by embedding 1-μm non-fluorescent polystyrene microspheres (i.e. scatterers) (Thermo Scientific, 5000 Series Polymer Particle Suspension, refractive index = 1.5979) into the phantom. The background fluorescence is introduced by 1.1-μm green fluorescent microspheres (Thermo Scientific, Fluoro-Max Dyed Green Aqueous Fluorescent Particles) of a fixed density of 1.2×10⁶ particles / mL. The imaging
targets are 25-μm green fluorescent microspheres (Thermo Scientific, Fluoro-Max Green Dry Fluorescent Particles) of a fixed density of $1.5 \times 10^4$ particles / mL. The background medium is the clear resin (FormLabs, #RS-F2-GPCL-04, refractive index is approximately 1.5403) for ease of fabrication. One caveat of this recipe is that the anisotropy factor $g$ of the phantoms is 0.965 due to the small refractive index contrast, which is larger than the commonly reported values for biological tissues (~0.9). This can result in worse background fluorescence in the raw measurements as compared to the case with smaller $g$ values (38).

Different amounts of non-fluorescent scatterers are added to the eight different phantoms with a micropipette (Thermo Scientific, Fisherbrand Elite Adjustable Volume Pipette, #FBE00100). Specifically, 0, 8, 16, 32, 64, 128, 256, and 512 µL of scatterer suspension (10% volume concentration) are added to 2 mL of clear resin, where 0 stands for the control “non-fluorescent scatter-free” phantom. Correspondingly, the rest of the seven phantoms contain $7.6 \times 10^8$, $1.5 \times 10^9$, $3.0 \times 10^9$, $5.9 \times 10^9$, $1.2 \times 10^{10}$, $2.2 \times 10^{10}$, and $3.9 \times 10^{10}$ particles / mL, respectively. After fully mixing the bead suspension with the clear resin, 0.1 mL from each mixed solution is then transferred to a 3D printed well (inner diameter 8 mm, height 2 mm, clear resin). Each phantom is then cured under a UV lamp. The pictures of the phantoms used in our experiments are shown in Fig. 5D.

The scattering mean free path $l_s$ of each phantom is estimated based on Eq. (3) derived from the Mie scattering theory (39):

$$l_s = \frac{2d}{3\Phi Q_s}, \quad (3)$$

where $d$ is the averaged diameter of the scatterers, and $\Phi$ is the volume fraction of the scatterers (calculated from the number of scatterers added to each phantom). $Q_s$ is the scattering efficiency factor calculated based on the Mie scattering calculator (40). For Phantom 1, we consider the 1.1-μm fluorescent beads as the main source of scattering and the corresponding $Q_s$ is 0.271. For Phantoms 2-8, we consider the 1.0-μm non-fluorescent beads as the main source of scattering and the corresponding $Q_s$ is 0.224. Accordingly, the scattering mean free paths $l_s$ for the eight phantoms are approximately 323.7, 7.51, 3.77, 1.9, 0.965, 0.497, 0.264, and 0.147 mm, respectively.
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Fig. 1. Single-shot 3D fluorescence Computational Miniature Mesoscope (CM²).
(A) The CM² combines a microlens array (MLA) optics and LED array excitation in a compact and lightweight platform. (B) A picture of the CM² prototype (the electric wires and the sensor driver are omitted). (C) The CM² measurement on 100-μm fluorescent particles suspended in clear resin. (D) The Projected view of the CM² reconstructed volume (7.0 mm × 7.3mm × 2.5mm) and three zoomed-in regions with orthogonal views. Scale bars: 100 μm.
Fig. 2. Characterization of the CM^2's 3D imaging capability. (A) The CM^2 produces axially de-correlated array PSFs to achieve optical sectioning. (B) The 3D MTF analysis shows that the CM^2 captures extended axial frequency information and improves the modulation contrast for high-resolution lateral features, both enlarging the system’s SBP. (C) The geometry for imaging a tilted fluorescent target placed at ~10 mm away from the CM^2. (D) The raw CM^2 measurement of the tilted fluorescent target. (E) The maximum intensity projections of the reconstructed volume (8.1 mm × 5.5 mm × 1.8 mm). The 6.2-μm features can be resolved as shown in the zoomed-in XY projection. The axial sectioning capability is characterized by the XZ projection, validating the feature size-dependent axial resolution.
**Fig. 3.** Single-shot 3D imaging of 10-μm fluorescent particles in a clear volume. (A) XY maximum intensity projection of the reconstructed volume spanning 5.7 mm × 6.0 mm × 1.0 mm. Top left insert: the raw CM$^2$ measurement. The FOV of the CM$^2$ is comparable to a 2× objective lens (red bounding box) and is ~25X wider than the 10× objective lens (blue bounding box). (B) Zoomed-in of the CM$^2$ 3D reconstruction benchmarked by the axial stack taken by a 10×, 0.25 NA objective lens. (C) The axial and lateral cross-sections of the recovered 10-μm particle. By comparing with the measurements from the standard widefield fluorescence microscopy, the CM$^2$ faithfully recovers the lateral profile of the particle and achieves single-shot depth sectioning. (D) The XZ cross-sectional view of a reconstructed fluorescent particle, as compared to the axial-stack acquired from the 2× and 10× objective lenses. Overall, the CM$^2$ provides a wide FOV and depth-sectioning comparable to that from a 10× 0.25NA objective lens using a single measurement.
Fig. 4. Imaging of fluorescent fibers on a curved surface. (A) The sample contains fluorescent fibers spread on a 3D printed curved surface that mimics the mouse cortex. (B) The depth-color coded projection of the reconstruction spanning a volume of ~7.8 mm × 4.9 mm × 0.9 mm. The orthogonal projections reveal the curvature of the sample. (C) The CM^2 resolves the fiber structures as verified by the cutline from the reconstruction compared to the measurement with a 2× 0.10 NA objective lens. (D) The depth sectioning of the CM^2 benchmarked by the widefield measurements from 2× 0.1 NA and 10× 0.25 objective lenses. The CM^2 accurately recovers in-focus fiber structures and suppresses out-of-focus fluorescence.
Fig. 5. Imaging of scattering phantoms. (A) A background subtraction procedure is devised to remove the slow varying background before performing the 3D deconvolution. (B) Quantitative evaluation of the CM² performance under bulk scattering and strong background fluorescence. The image quality of the raw measurement is quantified by the SBR. The reconstructed depth range is measured to quantify the CM²’s axial imaging capability. When the scattering is weak, the reconstructed depth range is primarily limited by the low SBR due to background fluorescence. As the scattering increases and \( l_s \) approaches the DOF of the CM², the reconstructed range reduces to the superficial layer and quantitatively bounded by the finite axial resolution. The error bars represent measurements from multiple sub-FOVs. \( l_s \) for Phantom 1 and 2 are 323.7 mm, 7.5 mm, respectively, and are omitted in the plot for better visualization. (C) The XZ maximum intensity projections of the reconstruction across eight phantoms with different scattering densities. The dashed line in each sub-figure indicates the top surface of each phantom. (D) The images of the scattering phantoms used in the experiments.
Fig. 6. Imaging of scattering sample with a curved surface. (A) The illustration of the scattering sample ($l_s \sim 264 \, \mu m$) with a curved surface and the CM$^2$ raw measurement. (B) The depth-coded maximum intensity projections of the CM$^2$ reconstruction recovers particles in the superficial layer of the curved surface. (C) The comparison between the CM$^2$ reconstruction and the widefield fluorescence measurements (10×, 0.25 NA and 20×, 0.40 NA) verifies that the CM$^2$ correctly reconstructs the emitters in the superficial layer.