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Confocal-inspired computational fluorescence microscopy via transfer point spread function

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Abstract

Laser scanning confocal microscopy provides optical sectioning but is limited by slow acquisition and hardware complexity. Widefield fluorescence microscopy offers high-throughput, low-cost imaging, yet global illumination generates substantial out-of-focus and multiply scattered background. Here we present computational confocal fluorescence microscopy (CCFM), a physics-grounded framework that leverages a transfer point spread function (TPSF) to reconstruct confocal-like volumes from widefield data. CCFM models widefield imaging as a cascaded process in which scattering-mediated redistribution is followed by system blur, enabling reconstruction beyond standard deconvolution. After a single calibration from paired widefield and confocal volumes, the TPSF can be reused for subsequent widefield imaging under matched imaging conditions and preprocessing strategy, without hardware modification. Across diverse specimens and imaging configurations, CCFM suppresses scattering background and restores optical sectioning beyond standard deconvolution, enabling confocal-like dynamic three-dimensional imaging at widefield speed.

1 Introduction

Fluorescence microscopy is a central tool in biological imaging because it enables molecularly specific visualization of cellular and subcellular structures [1]–[5]. Among its many implementations, laser scanning confocal microscopy (LSCM) and widefield fluorescence microscopy are two widely used imaging modalities that offer distinct trade offs in volumetric imaging. LSCM achieves optical sectioning through localized excitation and pinhole based detection, providing strong rejection of out of focus background and high contrast three dimensional imaging [6]–[11]. However, because LSCM relies on sequential point by

point excitation and detection, volumetric imaging is inherently time consuming, especially when large fields of view, high spatial sampling, or extended time series are required [8]-[11].

By contrast, widefield microscopy uses parallelized detection to enable rapid image acquisition with low hardware complexity and broad experimental accessibility. Its performance, however, deteriorates substantially in thick and scattering specimens because global illumination excites fluorophores throughout the sample volume, causing each recorded pixel to contain a mixture of in focus signal and multiply scattered out of focus background [12]-[15]. As a result, widefield imaging lacks the optical sectioning capability that is central to confocal microscopy, leaving an unresolved gap between imaging throughput and sectioning performance.

To recover optical sectioning from widefield data, computational deconvolution has long been regarded as a promising solution [16]-[22]. However, most conventional formulations assume that the dominant degradation arises from the imaging system itself [23],[24], while overlooking the preceding redistribution of the emission field by tissue scattering under global illumination [25]-[28]. As a result, conventional deconvolution removes only the blur imposed by the optical system, leaving unaddressed the preceding scattering induced degradation that has already redistributed the effective emission field. Emerging deep-learning strategies also attempt to learn mappings from widefield to LSCM modalities [29]-[31], but they typically rely on end-to-end modality transformations, thereby absorbing the missing physical relationship implicitly into data-driven models and often sacrificing generalizability and interpretability while requiring large amounts of paired training data [32]-[34].

In thick and scattering specimens, the degradation in widefield microscopy is more accurately described as a cascaded physical process involving fluorescence emission and system blur. Under global illumination, fluorescence is excited throughout the specimen volume, and photon scattering redistributes the emission field before it reaches the imaging system, causing the resulting emission field to deviate from the intrinsic object distribution [35]. This redistributed field is then further blurred by the aperture-limited system [36]. Therefore, the fluorescence emission process and the system blur process are more appropriately treated separately. Under the key prior that the specimen can be approximated as a slowly varying isotropic medium within the imaging region, this upstream redistribution admits a convolutional representation, in which the effective emission field is given by the convolution of the intrinsic object with a scattering kernel. Therefore, recovering the intrinsic object distribution requires accurate and separate characterization of the two degradations in this cascaded process: the downstream system blur can be handled by established approaches, whereas the upstream scattering-mediated redistribution must be constrained by a physical

reference that approximates the intrinsic distribution within the sample’s own scattering environment.

Confocal microscopy relies on point excitation and pinhole detection to suppress out-of-focus and multiply scattered components at both the excitation and detection stages, allowing its observations to serve as a physical reference. Using paired widefield and confocal observations from routine biological samples as priors, together with pre-calibration for system-blur removal, we decouple an effective transfer operator during estimation of the pre-processed widefield volume. Defined relative to the confocal reference, this operator is formulated as a transfer point spread function (TPSF). On this basis, we introduce Computational Confocal Fluorescence Microscopy (CCFM), a computational imaging framework that uses the calibrated TPSF as a reusable operator to recover confocal-like intrinsic distributions from widefield images. After a one-time paired widefield-confocal calibration, subsequent experiments can be carried out entirely on a standard widefield microscope, enabling confocal-quality volumetric imaging without scanning hardware or mechanical spinning disks while preserving the high acquisition speed of widefield detection.

A TPSF calibrated from a readily available biological sample can be reused across a range of specimens under matched imaging conditions and consistent preprocessing, with higher reconstruction fidelity typically achieved in samples with more similar structural and scattering properties. In fluorescent bead experiments, CCFM drives widefield observations toward the diffraction limit, indicating recovery close to the native resolving power of the optical system. Built on rapid widefield acquisition, CCFM enables dynamic volumetric imaging of fast biological processes, including Brownian motion, vascular transport, ciliary beating and cell lysis, while maintaining confocal-inspired sectioning over time. Taken together, these results establish CCFM as a general computational route for enabling confocal-like optical sectioning and dynamic volumetric fluorescence imaging on standard widefield microscopes through TPSF.

2 Results

TPSF calibration, validation and dynamic deployment in CCFM

Widefield fluorescence imaging in scattering specimens is more accurately described as a cascaded degradation process than as a single system-blur problem. Under global illumination, fluorescence from the intrinsic object distribution is first redistributed by tissue scattering and is then blurred by the diffraction-limited imaging system. We therefore write the widefield image formation as

$$G(\mathbf{r}) = [O(\mathbf{r}) \otimes \mathcal{K}_{scat}(\mathbf{r})] \otimes h_{sys}(\mathbf{r}) + N(\mathbf{r}) \quad (1)$$

where $G(\mathbf{r})$ denotes the measured widefield image, $O(\mathbf{r})$ denotes the intrinsic fluorescence distribution, $\mathcal{K}_{scat}(\mathbf{r})$ denotes the scattering kernel, $h_{sys}(\mathbf{r})$ denotes the system response, and $N(\mathbf{r})$ denotes the measurement noise.

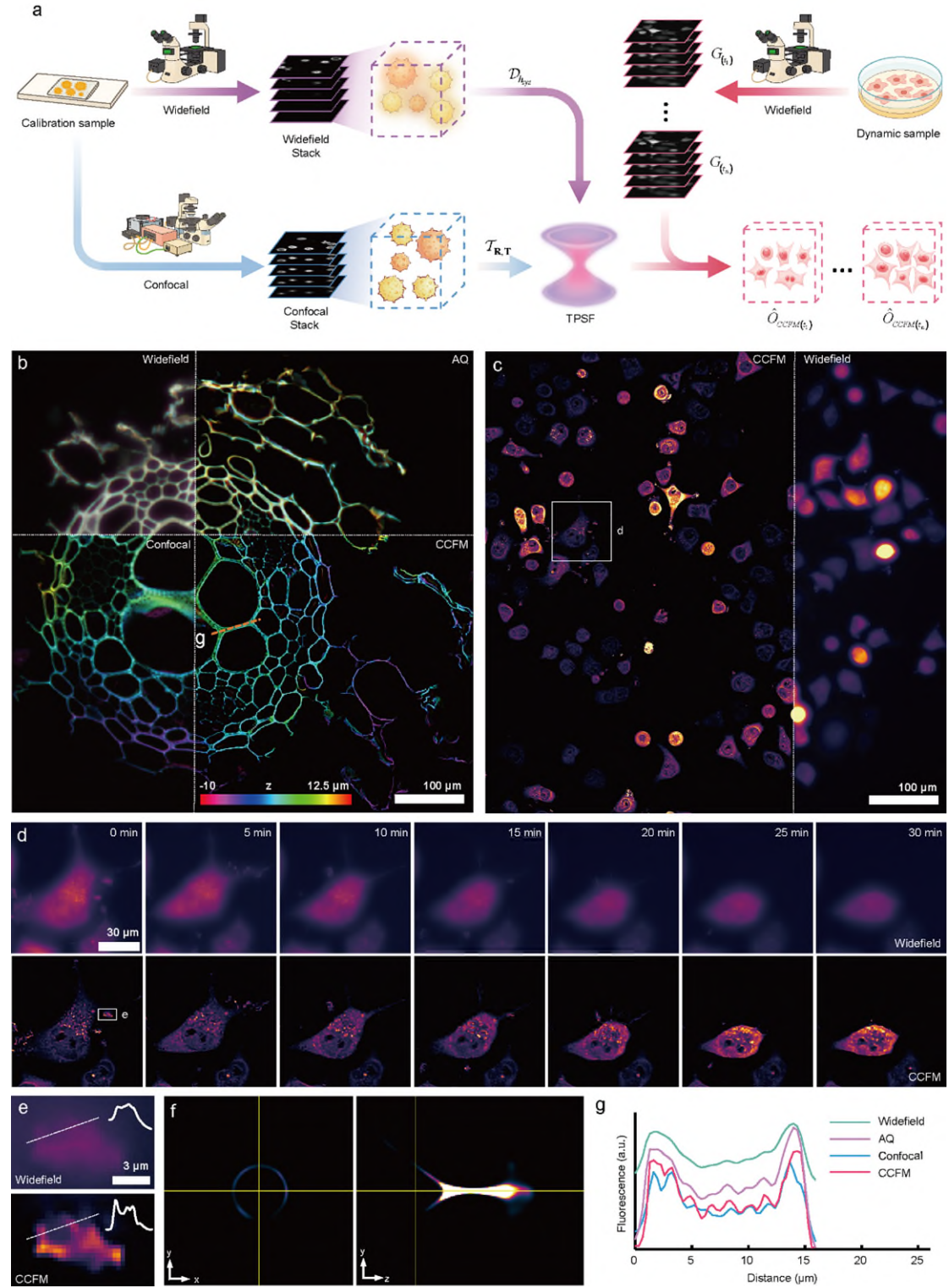


Fig. 1 | Calibration, validation and dynamic deployment of CCFM via a transfer point spread function. a,

Schematic of the CCFM workflow. Paired widefield and confocal volumes acquired from a static reference specimen were spatially registered and used to calibrate the transfer point spread function (TPSF). The calibrated TPSF was then reused to reconstruct confocal-like volumes from subsequent widefield measurements acquired under the same imaging conditions. **b**, Maximum-intensity projections of a transverse *Ranunculus* root section, showing raw widefield, AutoQuant X3 (AQ), confocal and CCFM images. **c**, Maximum-intensity projections of HeLa cells at a representative time point, comparing raw widefield and CCFM images. **d**, Enlarged views of the region of interest marked in **c** at different time points, with raw widefield images shown on top and CCFM reconstructions on the bottom. **e**, Enlarged view of ROI2 in **d**, with fluorescence intensity profiles across the cell boundary. **f**, xy and yz views of the calibrated TPSF. The xy slice is shown at $z = -23 \mu\text{m}$. **g**, Normalized fluorescence intensity profiles along Line 1 in **b**.

Global illumination interacts with biological tissue to induce the first stage of degradation. Fluorescence emitted from the intrinsic object propagates through the surrounding medium before entering the imaging system, where scattering gives rise to a scattered field composed of ballistic and snake photons. Under the slowly varying isotropic approximation, the local response form of the scattered field generated by each object point can be described by a scattering kernel $\mathcal{K}_{scat}$, which characterizes how point-source fluorescence is spatially redistributed by the medium before imaging. Accordingly, the first stage of degradation is written as the convolution of the intrinsic object distribution $O(\mathbf{r})$ with $\mathcal{K}_{scat}$ [14],[26],[35](Supplementary Notes 1-2). Subsequently, the resulting field then serves as the input to the imaging system and undergoes a second stage of degradation through spatial convolution with the diffraction limited system point spread function (PSF) h_{sys} , yielding the detected widefield image $G(\mathbf{r})$ [36].

Conventional deconvolution algorithms invert only the system PSF h_{sys} in an attempt to recover the intrinsic object distribution from $G(\mathbf{r})$ [37],[38]. Under the cascaded model described above, however, this operation merely removes the outer optical blur and does not directly recover the intrinsic object, but yields a pre-processed intermediate volume, denoted here as the effective redistribution estimate $\hat{\mathcal{E}}(\mathbf{r})$.

$$\hat{\mathcal{E}}(\mathbf{r}) = \mathcal{D}_{h_{sys}}(G(\mathbf{r})) \quad (2)$$

$\mathcal{D}_{h_{sys}}(\bullet)$ denotes a non-blind deconvolution operator with respect to the known system point spread function h_{sys} . Although this operation suppresses system-induced blur, the redistributed background and volumetric crosstalk produced during the first stage of degradation remain in $\hat{\mathcal{E}}(\mathbf{r})$, leaving structural details obscured [15],[39]. Recovering the intrinsic object therefore requires a further inverse step to decouple this scattering-induced degradation.

To further decouple scattering-induced degradation and enable confocal-like imaging from

widefield data, we developed a workflow using a static reference specimen (Fig. 1a and Supplementary Fig. 1). Given that the acquired confocal volume C_{calib} and widefield volume G_{calib} originate from distinct coordinate systems, they cannot be directly compared. We therefore apply a rigid spatial registration operator $\mathcal{T}_{\mathbf{R},\mathbf{T}}$ to the confocal data and obtain the registered confocal reference, yielding $C_{reg} = \mathcal{T}_{\mathbf{R},\mathbf{T}}(C_{calib})$. In parallel, we pre-process the calibration widefield volume G_{calib} by non-blind deconvolution and obtain the corresponding effective redistribution estimate, $\hat{\mathcal{E}}_{calib} = \mathcal{D}_{h_{sys}}(G_{calib})$.

With C_{reg} and $\hat{\mathcal{E}}_{calib}$ obtained, the calibration relation reduces to a single convolution governed by TPSF:

$$\hat{\mathcal{E}}_{calib}(\mathbf{r}) \approx C_{reg}(\mathbf{r}) \otimes h_t(\mathbf{r}) \quad (3)$$

We extract h_t by solving this inverse problem. Under the given imaging condition, h_t serves as an effective transfer operator linking the registered confocal reference C_{reg} to the effective redistribution estimate $\hat{\mathcal{E}}_{calib}$. To ensure mathematical robustness, estimation of h_t is formulated as a maximum likelihood estimation (MLE) problem. Given the intrinsic photon shot noise in fluorescence microscopy, we adopt an iterative Richardson Lucy algorithm based on Poisson statistics [37],[38] to approximate the optimal solution. The update rule is derived as follows:

$$h_t^{(k+1)}(\mathbf{r}) = h_t^{(k)}(\mathbf{r}) \cdot \left[\left(\frac{\hat{\mathcal{E}}_{calib}(\mathbf{r})}{C_{reg}(\mathbf{r}) \otimes h_t^{(k)}(\mathbf{r})} \right) \otimes C_{reg}^\dagger(\mathbf{r}) \right] \quad (4)$$

Here, k denotes the iteration index, and $C_{reg}^\dagger(\mathbf{r}) = C_{reg}(-\mathbf{r})$ represents the adjoint operator of the confocal volume, corresponding to spatial reversal. Because the theoretical or measured PSF used in pre-processing does not fully match the true system response under the sample environment, system blur cannot be completely removed in the first deconvolution step. The converged TPSF therefore captures not only the scattering kernel, but also the residual system response left by this imperfect deconvolution (Fig. 1f and Extended Data Fig. 2b–d, Supplementary Notes 3).

Once extracted, TPSF can subsequently be deployed in dynamic imaging scenarios (Fig. 1c). By first transforming high-speed widefield data into effective redistribution estimates and then reconstructing computational confocal volumes using TPSF, the framework achieves high-fidelity optical sectioning without the requirement for synchronized hardware scanning.

We validated CCFM on a 20 μm thick transverse section of *Ranunculus* root. Maximum-intensity projections showed that CCFM suppressed the out-of-focus background present in the

raw widefield image and recovered structural contrast in closer agreement with the confocal reference (Fig. 1b and Extended Data Fig. 1). Quantitative analysis of intensity profiles across representative structural features is shown in Fig. 1g. The profiles reconstructed by CCFM overlap closely with the measured confocal signal, whereas the widefield profile remains elevated owing to irreducible scattering background. These results confirm that the derived h_t provides an effective operator for suppressing widefield background and recovering confocal-like optical sectioning.

The morphology of h_t itself (Fig. 1f and Extended Data Fig. 2b-c; Supplementary Figs. 2 and 4) departs from the pure scattering kernel observed in simulation (Supplementary Figs. 5e), particularly through ring-like structures in the out-of-focus region. The experimentally decoupled TPSF cannot be explained by the pure scattering kernel alone, but is jointly shaped by the scattering kernel and the residual system response left after the first deconvolution; the ring-like structures in the out-of-focus region mainly indicate the latter, whereas the strong cross-sample similarity likely arises largely from the shared residual system response, and the improved adaptation on related specimens further indicates an additional contribution from the sample-dependent scattering kernel (Supplementary Figs. 2-4).

Finally, we demonstrate the application of CCFM in dynamic imaging, a regime in which confocal is typically constrained by acquisition speed. Rapid contraction dynamics of HeLa cells were imaged over a 30 min period (Fig. 1c-e). By deploying the TPSF to high-frame-rate widefield acquisitions, we reconstruct a CCFM time series. The resulting reconstructions clearly resolve subcellular contraction motions and boundary deformations that are indistinguishable in the raw widefield data because of out of focus blur (Fig. 1e). These results establish that CCFM effectively endows a standard widefield microscope with confocal-like optical sectioning, enabling long term, high contrast three dimensional observation of living systems at temporal resolutions inaccessible to point scanning techniques.

Performance comparison of CCFM

Using the same TPSF calibrated on the 20 $\times$, 0.75 NA imaging platform, we next evaluated the performance of CCFM across specimens of increasing structural and optical complexity. In thin mouse lung sections (Fig. 2a-e), CCFM effectively eliminates the defocused haze inherent to widefield acquisition and reveals sharply defined alveolar structures with contrast comparable to confocal imaging. The capability of the method was further challenged using thick and optically transparent rotifer specimens of approximately 100 μm in thickness (Fig. 2f-j, Supplementary Video 1). Owing to global illumination, the raw widefield volumes exhibit pronounced axial smearing, and commercial deconvolution software AutoQuant X3 (AQ) fails to fully suppress. In contrast, CCFM successfully decouples the scattering background and

recovers clearer volumetric organization and internal organelle details. Orthogonal views (Fig. 2i) confirm that CCFM restores optical sectioning performance comparable to physical pinhole filtering and effectively corrects axial elongation artifacts.

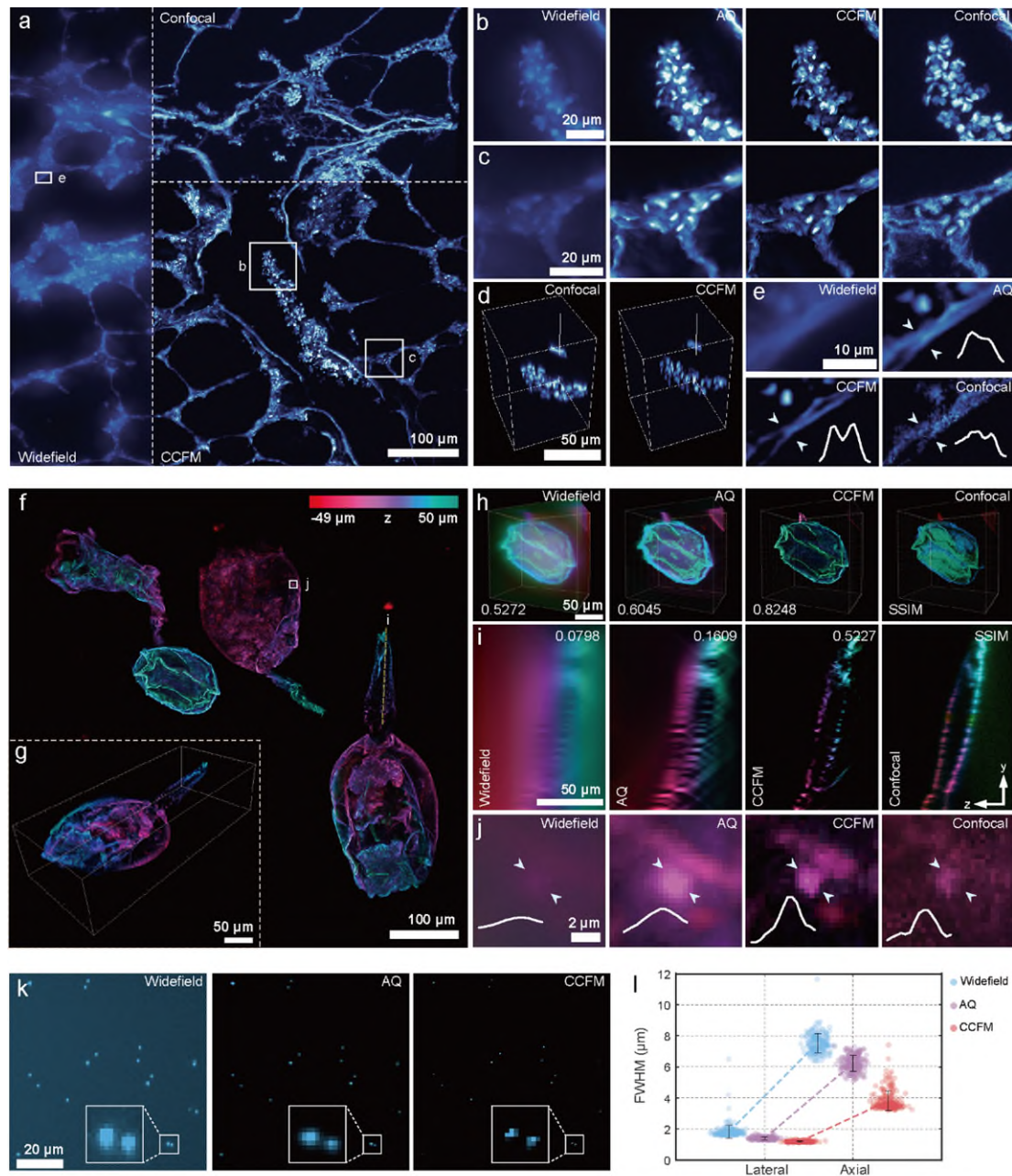


Fig. 2 | Performance of CCFM across specimen complexity and toward the diffraction limit. All data were acquired with a 20 $\times$, 0.75 NA objective. **a–e**, Validation on thin mouse lung sections. **a**, Large-field-of-view maximum-intensity projection comparing raw widefield, confocal and CCFM images. **b,c**, Enlarged views of the regions of interest indicated in **a**, comparing widefield, AQ, CCFM and confocal images. **d**, Three-dimensional volume rendering of the region shown in **b**. **e**, Enlarged view of region **e** in **a**, with fluorescence intensity profiles across representative structural edges. **f–j**, Validation on rotifer specimens of approximately 100 μm thickness. **f**, Color-coded depth projection of a rotifer specimen. **g**, Three-dimensional volume rendering of an individual rotifer. **h**, Enlarged volume view of the rotifer body. **i**, Orthogonal cross-sectional views along Line in **f**, comparing widefield, AQ, CCFM and confocal images. **j**, Enlarged view of region **j** in **f**, with corresponding fluorescence intensity profiles.

k,l, Quantitative resolution analysis using 500 nm fluorescent beads. **k**, Representative bead images from raw widefield data and the corresponding AQ and CCFM reconstructions. **l**, Statistical comparison of lateral and axial full width at half maximum values. Center lines and error bars indicate mean $\pm$ s.d.

To quantify this resolution enhancement, we imaged 500 nm fluorescent beads (Fig. 2k, l). Statistical analysis of the full-width at half-maximum (FWHM) reveals that while AQ provides limited axial improvement over the raw input, CCFM achieves markedly tight axial confinement, reducing the axial FWHM by approximately fourfold compared to AQ. These results demonstrate that the TPSF-based inverse operation drives the recovered axial confinement closer to the diffraction limit of the optical system.

Consistent behavior was further observed in additional scattering specimens, including butterfly leg and pollen samples (Extended Data Figs. 3 and 4, Supplementary Video 2-4). In these datasets, CCFM again outperformed standard deconvolution by more effectively removing scattering background and revealing structural features that remained obscured in the widefield and AQ reconstructions.

Performance of CCFM across high-NA and multicolor imaging configurations

To further assess the generality of CCFM, we first imaged stained transverse sections of Musa stems using a 40 $\times$ 0.9 NA objective (Fig. 3a–e). In the depth-encoded projections and orthogonal views (Fig. 3a–c), CCFM suppressed out-of-focus background relative to raw widefield imaging and recovered vascular structures with contrast in closer agreement with the confocal reference. Intensity profiles (Fig. 3e) further showed sharper structural transitions and improved signal-to-noise characteristics.

We next evaluated the framework on thin, low-signal specimens by imaging fixed bovine pulmonary artery endothelial (BPAE) monolayers (Fig. 3f,g). Owing to the limited photon budget of these cellular monolayers, the confocal images exhibit pronounced photon noise (Fig. 3g, right). In contrast, CCFM provided cleaner visualization of the F-actin and microtubule networks while preserving strong signal intensity, avoiding the noise penalty associated with point-scanning detection under low-light conditions. A similar trend was observed in specimens with large intensity variations. In approximately 15 μ m thick flower bud sections (Extended Data Fig. 5), the confocal reference showed saturation and photon noise in high-intensity regions, whereas CCFM produced more balanced reconstructions and resolved fine structural details such as bilayer cell walls (Extended Data Fig. 5c).

We further tested CCFM in high-NA volumetric imaging using a 100 $\times$, 1.4 NA objective (Fig. 3h–k). Under these conditions, the shallow depth of field leads to severe defocus blur in

widefield imaging, obscuring internal tissue organization in the volume renderings (Fig. 3j,k, left). CCFM reconstruction markedly reduced this blur and recovered clearer three-dimensional organization of the cell walls.

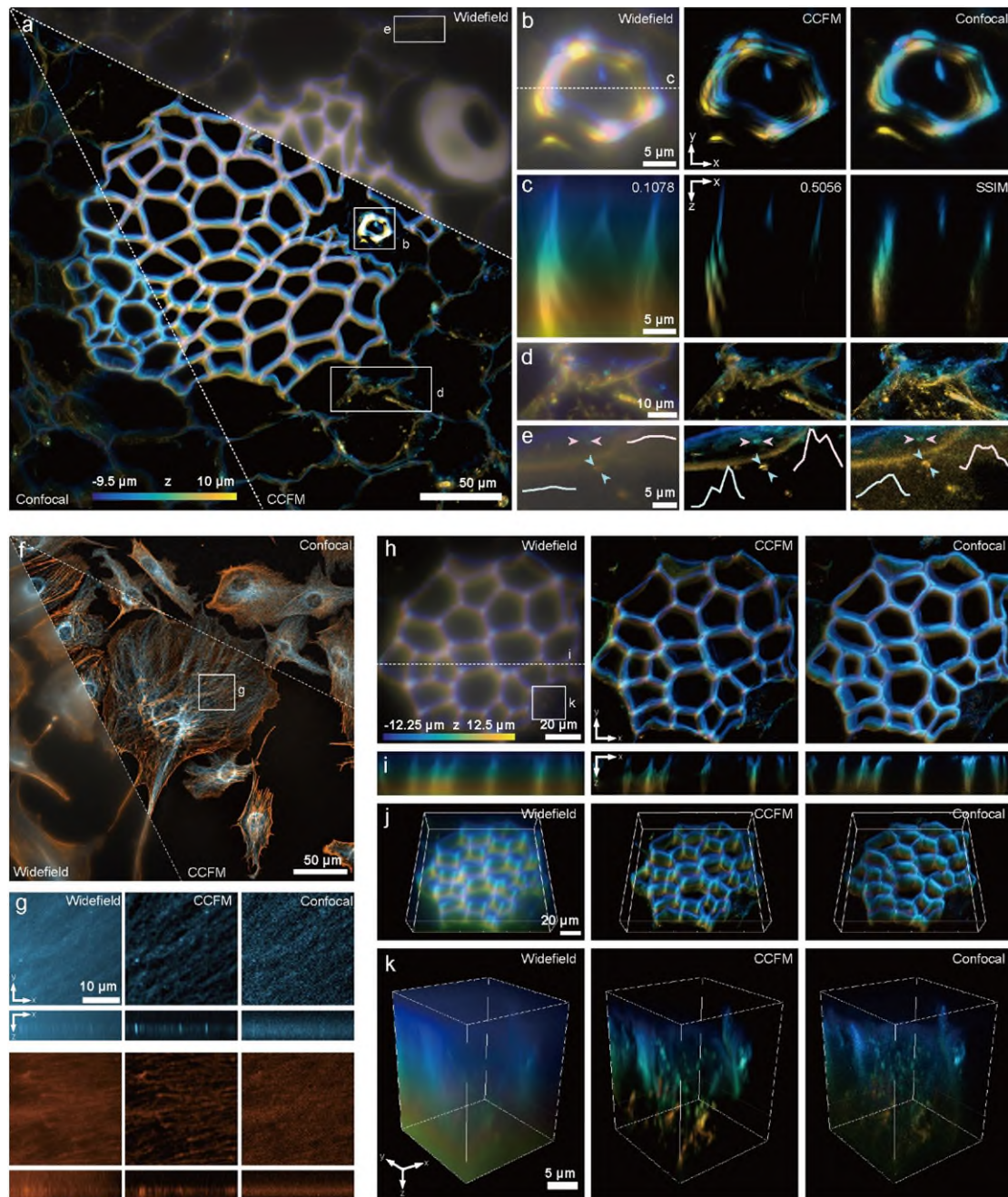


Fig. 3 | Performance of CCFM across high-NA and multicolor imaging configurations. a–g, Data acquired with a 40 $\times$, 0.9 NA air objective. **a–e,** Imaging of banana stem sections. **a,** Large-field-of-view color-coded depth projection comparing confocal, raw widefield and CCFM images. **b,** Enlarged view of the region marked in **a**. **c,** Orthogonal cross-sectional view along the dashed line in **b**, comparing widefield, CCFM and confocal images. **d,** Enlarged view of ROI **d** in **a**. **e,** Fluorescence intensity profiles across representative fine structures indicated by arrows. **f,g,** Dual-color imaging of bovine pulmonary artery endothelial cells labeled for F-actin in orange and microtubules in blue. **f,** Maximum-intensity projections comparing widefield, CCFM and confocal images. **g,** Enlarged views of the ROI in **f**, with lateral views on top and axial views on bottom. **h–k,** Data acquired with a 100 $\times$,

1.4 NA oil-immersion objective. **h**, Color-coded depth projection of a banana stem section comparing widefield, CCFM and confocal images. **i**, Orthogonal view along the dashed line in **h**, comparing widefield, CCFM and confocal images. **j**, Three-dimensional volume rendering of the region shown in **h**. **k**, Enlarged three-dimensional rendering of the ROI in **h**.

Finally, we quantified the resolution enhancement achieved by CCFM using 100 nm fluorescent beads imaged with a 40 $\times$, 0.9 NA objective (Extended Data Fig. 6). Comparative analysis against Richardson–Lucy (RL) deconvolution showed that CCFM more effectively reduced blur-induced axial elongation (Extended Data Fig. 6c). The statistical distribution of FWHM further showed that CCFM shifted the reconstructed bead response closer to the diffraction limit (Extended Data Fig. 6d,e), indicating that TPSF-based reconstruction improves widefield recovery beyond standard deconvolution under high-NA imaging conditions.

Taken together, these results show that CCFM extends from standard 20 $\times$ imaging to higher-NA and multicolor experiments. In practice, different magnifications correspond to different imaging systems and therefore require different TPSFs, whereas multicolor channels acquired within the same imaging system can share a TPSF because wavelength-dependent system blur is removed in the initial deconvolution step.

In addition, reprocessing of an open source *C. elegans* embryo dataset [17] further confirms the generality of CCFM (Extended Data Fig. 7). Compared with the Deconvolf algorithm [22], CCFM more effectively suppresses residual volumetric haze across all three spectral channels (Extended Data Fig. 7a–c), demonstrating that CCFM can serve as a general purpose post processing tool to substantially enhance existing widefield microscopy datasets.

Dynamic imaging of rapid biological processes

In dynamic imaging, achieving high contrast at high temporal resolution remains challenging. CCFM enables recovery of optical sectioning from widefield acquisitions, thereby facilitating high speed time lapse and volumetric imaging of fast biological processes. We first validated this capability by imaging Brownian motion of autofluorescent *Chlorella* (Fig. 4a–d, Supplementary Video 5). Temporal maximum intensity projections (Fig. 4a, b) visualize the aggregate motion trajectories, clearly distinguishing stationary cells (white) from the motile population (colored tails). To analyze motion artifacts, we extracted time unfolded plots along the trajectory of a representative cell indicated by the dashed line in Fig. 4b (Fig. 4c). In widefield images, rapid cellular motion is conflated with scattering, producing continuous blurred streaks, whereas CCFM resolves discrete cell morphologies at each time point, enabling reliable identification of individual cells within dense moving clusters (Fig. 4d).

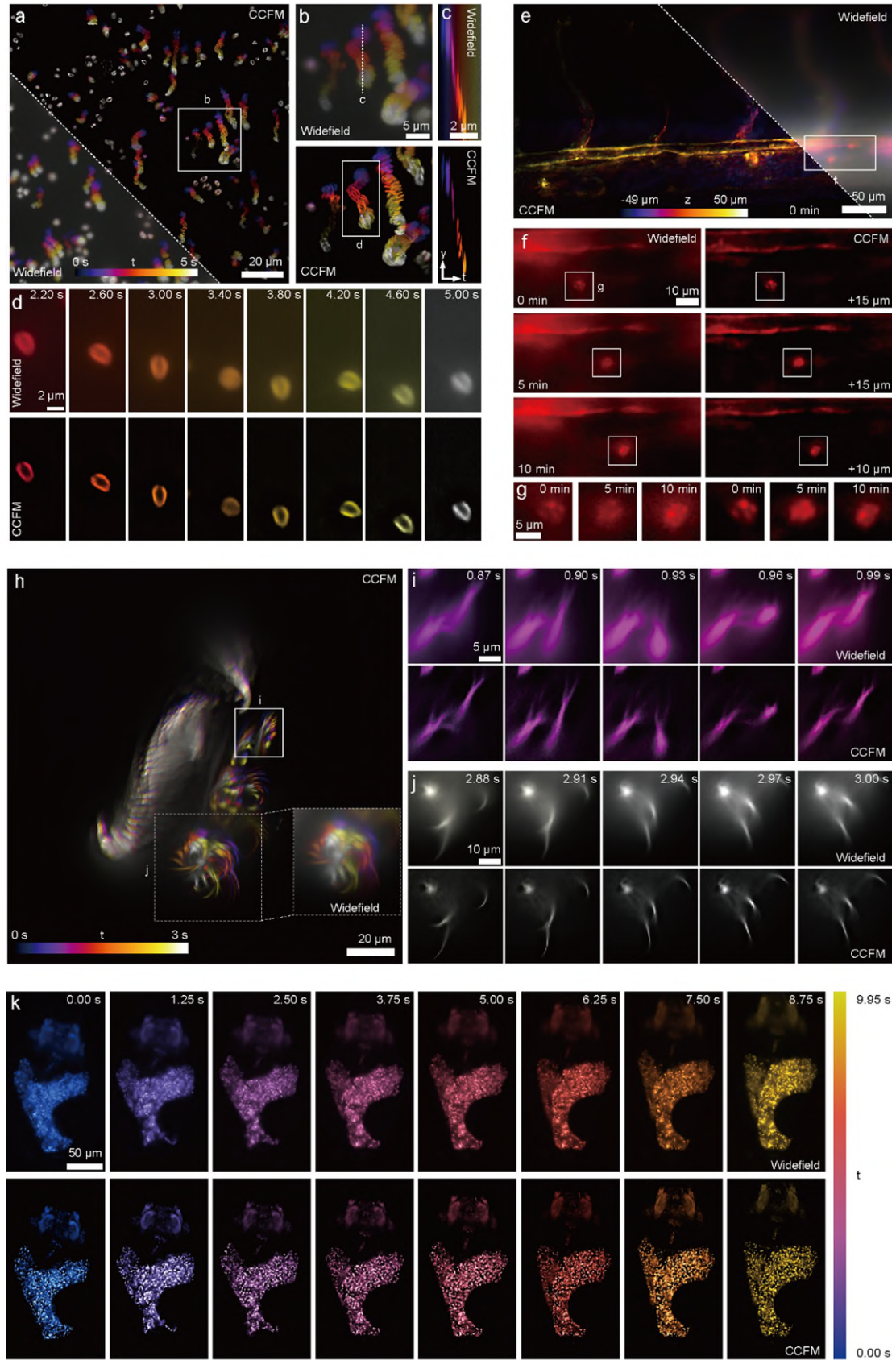


Fig. 4 | Dynamic imaging of rapid biological processes with CCFM. **a–d**, Data acquired with a 100×, 1.4 NA oil-immersion objective. **a**, Time color-coded trajectory of autofluorescent *Chlorella* from $t=0$ to 5 s. **b**, Enlarged view of the region marked in **a**. **c**, Time-unfolded kymographs extracted along the dashed trajectory in **b**. **d**, Time series

comparison for the region shown in **b**. **e–k**, Data acquired with a 20 $\times$, 0.75 NA air objective. **e–g**, Volumetric monitoring of the vasculature in transgenic zebrafish Tg(fli1a:RFP). **e**, Color-coded depth projection of the CCFM reconstruction. **f**, Time series showing a motile fluorescent object circulating within the vessel lumen in **e**. **g**, Enlarged views of the object in **f** at three time points, 0, 5 and 10 min. **h–j**, High-speed recording of ciliary beating in DiI-stained Paramecium. **h**, Time color-coded projection of ciliary motion from $t=0$ to 3 s. **i,j**, Time series snapshots of the cilia marked in **h**, comparing widefield images on top and CCFM reconstructions on bottom. **k**, Time series capturing osmotic stress-induced membrane rupture and subsequent intracellular content dispersion in Paramecium from $t=0$ to 9.95 s, with widefield images on top and CCFM reconstructions on bottom.

We then applied CCFM to volumetric monitoring of the vasculature in transgenic zebrafish Tg(fli1a:RFP) using a 20 $\times$, 0.75 NA objective (Fig. 4e–g). The reconstructed depth projection resolved the three-dimensional vascular architecture, and the subsequent time series revealed a motile fluorescent object circulating within the vessel lumen (Fig. 4e,f). Enlarged views at 0, 5 and 10 min further showed that CCFM could follow its motion over time while maintaining volumetric contrast in the surrounding vessel structure (Fig. 4g).

We further applied CCFM to high speed imaging of ciliary beating in Paramecium (Fig. 4h–j). The rapid, coordinated motion of cilia typically forms metachronal waves. In widefield recordings, integration of signals from overlapping focal planes blurs these beating cilia into continuous, indistinct bands (Fig. 4i,j, top). Temporal unfolding along regions of interest (Fig. 4i,j) shows that CCFM effectively disentangles these overlapping signals, resolving the smeared patterns into discrete whip like filaments and revealing the metachronal wave dynamics that are otherwise obscured by motion blur.

Finally, we captured the critical state of a Paramecium during cell lysis, moments before complete disintegration by osmotic mismatch (Fig. 4k, Supplementary Video 6). After membrane rupture, rapid dye influx leads to severe deformation, and the widefield sequence becomes dominated by scattering artifacts. CCFM resolves dense intracellular components throughout the collapse, demonstrating robust contrast preservation during extreme structural dynamics.

3 Discussion

Our work builds on a cascaded degradation model of widefield fluorescence imaging, in which scattering-mediated redistribution before imaging and system blur during detection are treated separately, enabling recovery of confocal-like distributions through calibration-based decoupling. Experimental results show that this framework enables robust confocal-like reconstruction across specimens, drives recovery closer to the diffraction limit in fluorescent bead experiments, and maintains optical sectioning in dynamic imaging. CCFM therefore provides a physics-grounded computational route by which standard widefield fluorescence

microscopy can achieve interpretable and reusable confocal-like optical sectioning through one-time calibration.

More specifically, CCFM should be understood as a physics-calibrated analytical computational imaging framework. Rather than relying on an end-to-end modality transformation, it estimates an explicit transfer point spread function from paired widefield and confocal observations after consistent preprocessing and spatial registration, and then uses this calibrated operator to reconstruct confocal-like volumes from subsequent widefield measurements acquired under matched conditions. This calibration-based design underlies both the interpretability of the method and its practical reusability.

While this reuse is robust under fixed imaging conditions, it should not be interpreted as strict sample invariance. Under the same imaging platform, TPSFs decoupled from distinct biological specimens remain highly consistent in their dominant three-dimensional structure, with much of this overall similarity arising from the shared residual system response, while sample dependence is manifested mainly as limited local variation associated with the scattering contribution (Supplementary Fig. 2). In practice, such variation may lead to modest differences in reconstruction fidelity across target specimens, and a TPSF derived from a structurally related specimen may provide a better match because its scattering contribution is more compatible with the target sample (Supplementary Fig. 3). The reuse of TPSF also depends on calibration consistency, because differences in the preprocessing used in the first deconvolution alter the residual system response retained in the subsequently decoupled TPSF (Supplementary Fig. 4).

Because CCFM is built on an explicit physical model, its underlying assumptions and practical boundaries need to be clearly stated. The method relies on the slowly varying isotropic approximation, under which emission redistribution can be approximated by a spatially invariant convolution process; this approximation may no longer hold in strongly heterogeneous or extremely thick samples. In addition, the TPSF is affected by sampling density and the specific imaging settings, so it generally needs to be recalibrated when these conditions change. At the implementation level, the method also depends on two practical requirements: accurate registration between widefield and confocal images, and a region of interest that fully covers the effective support of the scattering tail, since otherwise boundary artifacts can be introduced during deconvolution.

As an iterative reconstruction method based on an analytical model, CCFM is currently still limited in computational speed by the deconvolution process, so its further development will require not only faster reconstruction but also extensions of the model itself. On the computational side, future work may explore faster iterative strategies, approximate inversion

schemes, or data-driven reconstruction combined with physical constraints to improve efficiency while preserving physical interpretability. On the modeling side, it will be important to develop imaging models that can accommodate spatially varying and anisotropic scattering, thereby extending the method to more complex, thicker, and more heterogeneous biological samples. Along these directions, CCFM may further expand the potential of widefield fluorescence microscopy for high-throughput, long-term, and dynamic volumetric imaging.

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4 Methods

Theoretical foundation of scattering-mediated redistribution

To rigorously quantify the impact of tissue scattering on fluorescence imaging, we formulate a statistical physical framework grounded in mesoscopic wave optics. The spontaneous emission of a single fluorophore is modeled as an oscillating electric dipole located at $\mathbf{r}_0$ [40], whose complex electric field $E(\mathbf{r}, \omega)$ in an inhomogeneous medium with random refractive index fluctuations $\epsilon(\mathbf{r})$ satisfies the inhomogeneous Helmholtz equation [14],[41]:

$$(\nabla^2 + k^2\epsilon(\mathbf{r}))E(\mathbf{r}, \omega) = S(\mathbf{r}, \omega) \quad (5)$$

where the source term $S(\mathbf{r}, \omega) = p\delta(\mathbf{r} - \mathbf{r}_0)$ specifies the physical origin of the wave and the dipole moment p encodes the initial amplitude and orientation of the point emitter (Supplementary Note 1). Owing to the linearity of the wave equation, the instantaneous electric field at the detection point $\mathbf{r}$ is given by the product of the Green's function $G(\mathbf{r}, \mathbf{r}_0)$ and the source term, $E(\mathbf{r}) = G(\mathbf{r}, \mathbf{r}_0) \cdot p$. Physically, G fully records all possible multiple scattering paths between the source and the surface, together with their phase coherent superposition [35] (Supplementary Note 1).

In practice, fluorescence emission is continuous, and the observable quantity corresponds to an intensity averaged over the temporal incoherence of fluorescence and the microscopic randomness of biological tissue [26],[42]. Within statistical optics, the point source intensity is therefore expressed as $I_{point}(\mathbf{r}) = \langle |E(\mathbf{r})|^2 \rangle = \langle |G(\mathbf{r}, \mathbf{r}_0)|^2 \rangle \cdot |p|^2$. We define the scattering kernel $\mathcal{K}_{scat}$ as the expected value of this intensity propagator [26], $\mathcal{K}_{scat}(\mathbf{r} - \mathbf{r}_0) \equiv \langle |G(\mathbf{r}, \mathbf{r}_0)|^2 \rangle$. Under the slowly varying isotropic approximation, $\mathcal{K}_{scat}$ is approximately translation invariant and characterizes the spatial redistribution of point-source fluorescence before entering the imaging system (Supplementary Note 1). For a macroscopic object O composed of a large ensemble of mutually incoherent fluorophores, the effective scattering field $\mathcal{E}$ before entering the imaging system is the linear superposition of all point-source responses. By the principle of linear superposition, this process can be written as a convolution between the intrinsic object distribution and the scattering kernel [36], $\mathcal{E} = O \otimes \mathcal{K}_{scat}$ (Supplementary Note 2). This formulation abstracts the random wave process into a deterministic spatial redistribution model, providing the theoretical foundation for the cascaded convolution model and subsequent scattering decoupling.

Cascaded imaging model and residual system response

Having established the statistical foundation of the effective emission field $\mathcal{E}$, we next describe how this field is captured by the optical system and how intrinsic relationships arise

between different imaging modalities. Under widefield detection, the scattered fluorescence is further blurred by the system response under the sample environment, so that the recorded image can be written as

$$G(\mathbf{r}) = \mathcal{E}(\mathbf{r}) \otimes h_{sys}(\mathbf{r}) + N(\mathbf{r}) \quad (6)$$

where h_{sys} denotes the system response and N measurement noise. Combined with the scattering-mediated redistribution model $\mathcal{E} = O \otimes \mathcal{K}_{scat}$, the widefield formation process is described by the cascaded imaging model

$$G(\mathbf{r}) = [O(\mathbf{r}) \otimes \mathcal{K}_{scat}(\mathbf{r})] \otimes h_{sys}(\mathbf{r}) + N(\mathbf{r}) \quad (7)$$

which shows that widefield degradation arises from two successive stages: scattering-mediated redistribution before imaging and system blur during detection [14],[26] (Supplementary Note 4).

In practice, the system response used in the first deconvolution step is not obtained directly under the exact sample environment, but is typically approximated by a theoretical PSF or a PSF measured from fluorescent beads h_{pre} [41],[43] (Supplementary Note 3). Here, it is important to distinguish the ideal scattering field from its experimentally recovered counterpart: $\mathcal{E}$ denotes the ideal scattering field before entering the imaging system, whereas $\hat{\mathcal{E}}$ denotes its estimate obtained after widefield pre-processing.

Because h_{pre} is only an approximation to the system response under the sample environment, the first deconvolution step does not fully recover the ideal scattering field $\mathcal{E}$. Instead, the recovered quantity $\hat{\mathcal{E}}$ should be understood as an effective redistribution estimate that still retains a residual system response in addition to the scattering-mediated redistribution. We denote the corresponding residual component as the residual system h_r , which represents the system blur not completely removed by the first deconvolution.

Physical interpretation and calibration-based decoupling of the TPSF

For a static calibration specimen with sufficiently high signal-to-noise ratio, the noise term can be neglected during TPSF calibration. Under this condition, the measured widefield image is written as

$$G_{calib}(\mathbf{r}) = O_{calib}(\mathbf{r}) \otimes \mathcal{K}_{scat}(\mathbf{r}) \otimes h_r(\mathbf{r}) \otimes h_{pre}(\mathbf{r}) \quad (8)$$

After the first non-blind deconvolution with h_{pre} , the recovered effective redistribution estimate becomes

$$\hat{\mathcal{E}}_{calib}(\mathbf{r}) = O_{calib}(\mathbf{r}) \otimes [\mathcal{K}_{scat}(\mathbf{r}) \otimes h_r(\mathbf{r})] \quad (9)$$

Here, the bracketed term represents the composite effect that remains after the first deconvolution, jointly containing scattering-mediated redistribution and residual system response.

In the calibration setting, confocal imaging provides a physically constrained reference because point excitation and pinhole detection suppress much of the diffuse background introduced before widefield detection [5],[7]. After rigid registration into the same coordinate system, the confocal volume $C_{reg}(\mathbf{r})$ can therefore be used as a reference approximation to the intrinsic object distribution of the calibration specimen, giving $O_{calib}(\mathbf{r}) = C_{reg}(\mathbf{r})$ (Supplementary Note 5). Under this approximation, the composite effect retained in $\hat{\mathcal{E}}_{calib}(\mathbf{r})$ can be related to the confocal reference rather than directly to the unknown intrinsic object.

Substituting the confocal reference approximation into the recovered redistribution estimate gives

$$\hat{\mathcal{E}}_{calib}(\mathbf{r}) \approx C_{reg}(\mathbf{r}) \otimes [\mathcal{K}_{scat}(\mathbf{r}) \otimes h_r(\mathbf{r})] \quad (10)$$

Because the bracketed term now characterizes the transfer from the registered confocal reference to the recovered widefield redistribution estimate, we define it as the transfer point spread function h_t . The calibration relation is therefore written as

$$\hat{\mathcal{E}}_{calib}(\mathbf{r}) \approx C_{reg}(\mathbf{r}) \otimes h_t(\mathbf{r}) \quad (11)$$

Estimating h_t from the calibration relation constitutes an inverse problem with known input C_{reg} and observed output $\hat{\mathcal{E}}_{calib}$. We solve this equation using a dual Richardson–Lucy algorithm derived from a maximum likelihood estimation (MLE) framework under Poisson statistics [44] (Supplementary Note 6). The estimation of h_t was implemented using the dual Richardson–Lucy update given in Eq. (4). Unlike standard deconvolution, which iteratively updates the object, this dual formulation updates the kernel while holding the object fixed. To ensure physical validity, we enforce two constraints during optimization [45],[46]: nonnegativity, reflecting the probabilistic nature of photon counts, and energy conservation, $\int h_t(\mathbf{r}) d\mathbf{r} = 1$, ensuring that the kernel acts purely as a redistribution function without amplifying the total photon flux.

CCFM reconstruction via TPSF

To recover confocal equivalent structures O from widefield measurements, we implement a two step reconstruction strategy that sequentially inverts the underlying physical processes. For a target widefield measurement acquired under the same imaging condition, reconstruction begins with the same pre-processing used in calibration. Specifically, the

measured widefield image G is processed with the pre-processing PSF h_{pre} to obtain the effective redistribution estimate $\hat{\mathcal{E}}$ [16],[37],[38]. This estimate then serves as the direct input to the subsequent CCFM reconstruction, in which the calibrated TPSF h_t is used to recover the confocal-like object distribution.

Reconstruction of the object $\hat{O}_{CCFM}$ is achieved by solving the inverse problem

$$\hat{O}_{CCFM}(\mathbf{r}) = \hat{\mathcal{E}}(\mathbf{r}) \otimes^{-1} h_t(\mathbf{r}) \quad (12)$$

To ensure consistency between calibration and reconstruction, the same pre-processing strategy must be used in both stages. In particular, the pre-processing PSF h_{pre} applied during reconstruction should remain consistent with that used for TPSF calibration, so that the residual system response absorbed into h_t remains compatible with the recovered redistribution estimate $\hat{\mathcal{E}}$. Under matched imaging conditions and sampling settings, the calibrated TPSF can therefore be reused for subsequent widefield measurements to recover CCFM volumes.

Data preprocessing and spatiotemporal registration

Accurate kernel estimation requires voxel level alignment between widefield and confocal measurements. We implement a coarse to fine rigid registration protocol [47] to compensate for mechanical drift or chromatic misalignment (Supplementary Note 7). To address differences in axial sampling, typically $\Delta z_{WF} < \Delta z_{CF}$, the recovered effective redistribution estimate is downsampled after registration using cubic spline interpolation to match the geometric grid of the confocal data [48]. In addition, to prevent boundary artifacts and spectral ringing arising from truncation of the scattering halo [49], we ensure that the acquired field of view extends beyond the biological region of interest by more than the effective scattering radius, approximately 20 to 50 μm , thereby satisfying the Minkowski sum condition for valid convolution [50] (Supplementary Note 7).

Sample preparation

Biological samples and reagents

Commercial fixed slide specimens used in this study, including transverse sections of *Ranunculus* root, mouse lung tissue sections, *Rotifera*, butterfly legs and pollen grains from *Cucurbita* and *Lilium*, were purchased from Suzhou SAGA Optical Instrument Co., Ltd. Fixed bovine pulmonary artery endothelial cells (BPAE, F14781, Thermo Fisher Scientific) were labeled with multiple fluorophores. F actin was stained with Texas Red X phalloidin, microtubules were labeled using an anti bovine α tubulin mouse monoclonal antibody (236-10501) followed by BODIPY FL conjugated goat anti mouse IgG, and nuclei were

counterstained with DAPI. Hand cut sections of banana stem and flower buds were prepared in house and stained with a mixture of TRITC and FITC.

Live sample preparation

HeLa cells expressing red fluorescent protein were purchased from Shanghai Fuheng Biotechnology Co., Ltd. and passaged several times before imaging. Cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin streptomycin at 37 °C in a humidified incubator with 5% CO₂.

Transgenic zebrafish larvae expressing red fluorescent protein in vascular endothelium, Tg(fli1a:RFP), were obtained from Shandong Yixiyue Biotechnology Co., Ltd. Larvae at 24 to 36 hours post fertilization were selected for imaging. To minimize motion artifacts, larvae were anesthetized and embedded in 1% low melting point agarose in glass bottom dishes.

Paramecium cells were collected from autotrophic cultures and stained with the membrane dye DiI (Biosharp, China). A 1 mM stock solution prepared in anhydrous DMSO was diluted to approximately 1 μ M in culture medium. Cells were mixed with the dye solution at a 4:1 ratio, incubated in the dark for 10 min, washed, and immobilized in 1% carboxymethyl cellulose for imaging in the TRITC channel.

Resolution test targets

Red fluorescent silica microspheres with diameters of 500 nm and 200 nm, used for PSF characterization and resolution quantification, were purchased from Jiangsu Zhichuan Technology Co., Ltd. The stock suspensions were diluted with distilled water to an appropriate density to avoid aggregation and mounted on standard microscope slides.

System configuration and algorithm setup

Widefield microscopy

Widefield imaging was performed on an inverted fluorescence microscope (IX81, Olympus) equipped with a motorized z stage. Illumination was provided by a 100 W mercury short arc lamp (U-LH100HG, Olympus) with standard filter sets for DAPI, FITC/GFP and TRITC/RFP. Images were acquired using an sCMOS camera (ORCA-Flash4.0 LT, C11440-42U40, Hamamatsu; 2048 $\times$ 2048 pixels, 6.5 μ m pixel size). Three objectives were used to cover different spatial scales: UPLSAPO 20 $\times$ /0.75 (air), UPLSAPO 40 $\times$ /0.90 (air) and UPLSAPO 100 $\times$ /1.40 (oil).

Confocal microscopy

Confocal volumes were acquired using a laser scanning confocal microscope (TCS SP8, Leica Microsystems). The illumination sources included a 405 nm diode laser, a multiline argon ion laser (458, 476, 488, 496 and 514 nm) and a tunable white light laser (WLL, 470–670 nm).

Detection was performed using hybrid detectors and photomultiplier tubes, with the pinhole set to 1.0 Airy unit. To match the widefield resolution and sampling conditions, the corresponding objectives were used: HC PL APO 20×/0.75 (air), HC PL APO 40×/1.10 (water) and HC PL APO 100×/1.40 (oil).

Computational hardware

All computations, including TPSF derivation and iterative deconvolution, were carried out on a high performance workstation equipped with dual Intel Xeon Gold 5220 CPUs (72 cores in total), 256 GB RAM and an NVIDIA TITAN RTX GPU (4,608 CUDA cores, 32 GB memory), running MATLAB (R2023b, MathWorks).

Reference deconvolution algorithms

To benchmark the performance of TPSF based restoration, we performed comparative three dimensional deconvolution using established software. Commercial blind deconvolution was carried out with AutoQuant X3 (Media Cybernetics) featuring adaptive PSF estimation. Open source deconvolution was performed using DeconvolutionLab2 [17] (EPFL) and Deconvolf [22], with theoretical PSFs generated according to the optical parameters of each experiment including numerical aperture, refractive index and wavelength.

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Data availability

The minimum dataset required to interpret, verify and reproduce the findings of this study will be deposited in a public repository and made publicly available upon publication. Owing to the large size of the full raw imaging dataset, only the minimum dataset and representative raw data necessary to reproduce the main analyses will be publicly archived; additional raw data will be available from the corresponding author upon reasonable request.

Code availability

Custom code used to generate the main results in this study will be deposited in a public, versioned repository and made publicly available upon publication.

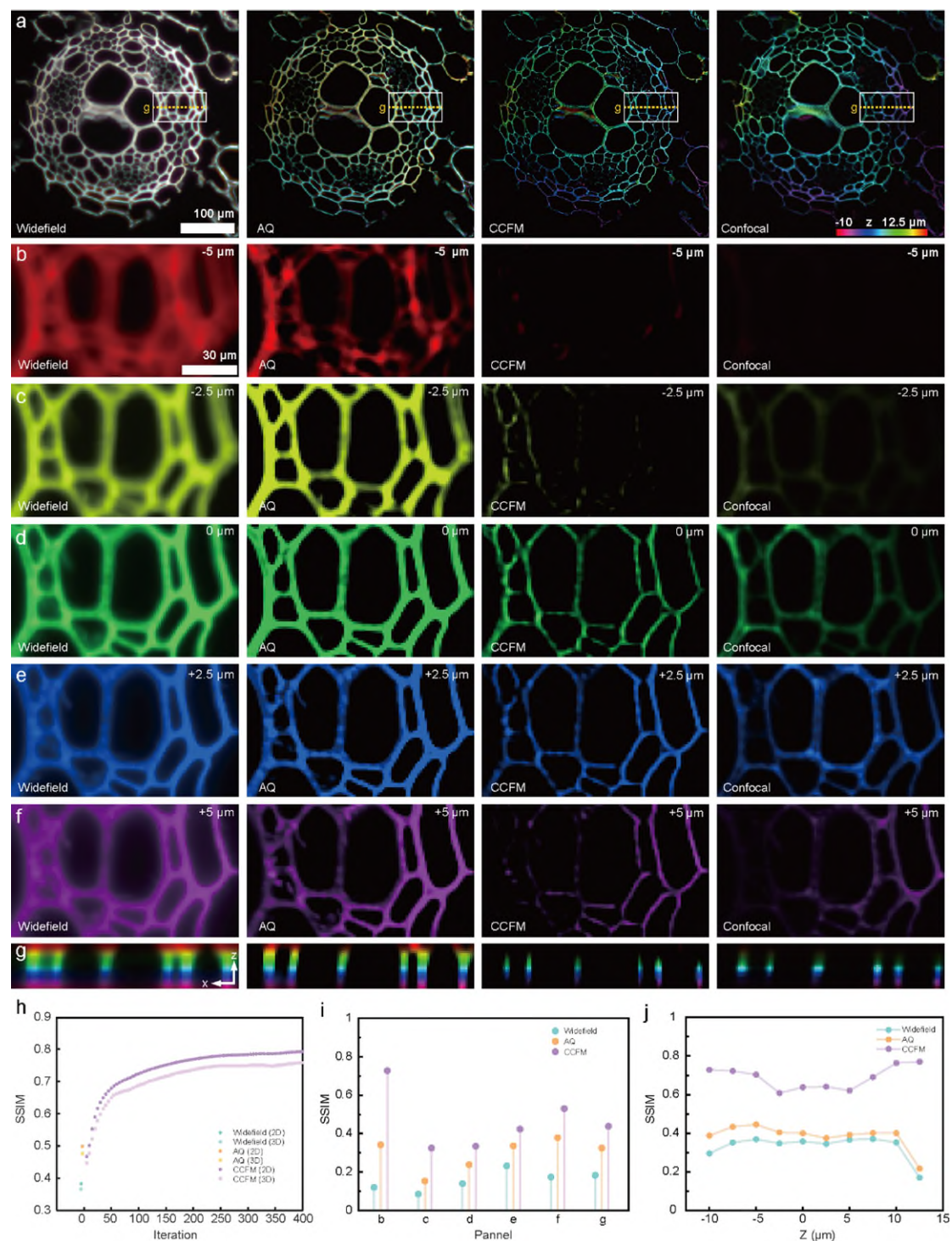
Conflict of interest

The authors declares no competing interests.

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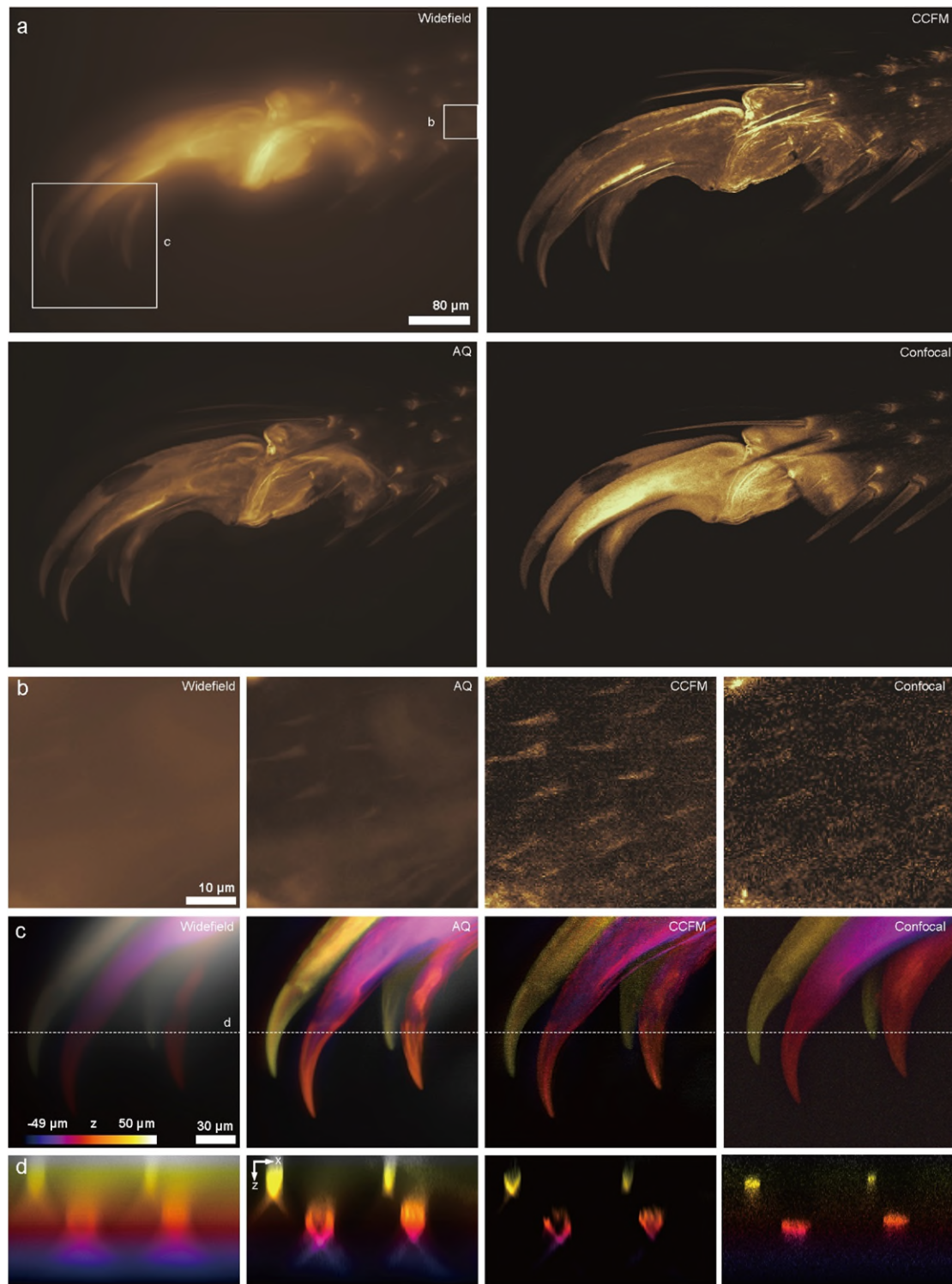
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Extended Data Fig. 1 | Axial slice fidelity and convergence analysis in transverse *Ranunculus* root sections. **a**, Color-coded depth projection of a transverse *Ranunculus* root section imaged with a 20 $\times$, 0.75 NA objective. Columns compare raw widefield, AQ, CCFM, and confocal. CCFM reconstruction shows clearer separation of structures at different depths and recovers contrast in closer agreement with the confocal reference. **b–f**, Single-plane z-slice comparisons corresponding to the regions marked in **a** over a depth range from $-5 \text{ } \mu\text{m}$ to $+5 \text{ } \mu\text{m}$. Notably, at $z = -5 \text{ } \mu\text{m}$ in **b**, where no physical structures are present in the focal plane, both widefield and AQ images exhibit strong out-of-focus artifacts arising from interplane crosstalk, whereas CCFM produces a signal-free background consistent with the confocal reference. **g**, Fluorescence intensity profiles across Line 1 in **b**, showing that CCFM closely reproduces the confocal response while suppressing background offset from adjacent planes. **h**, Convergence

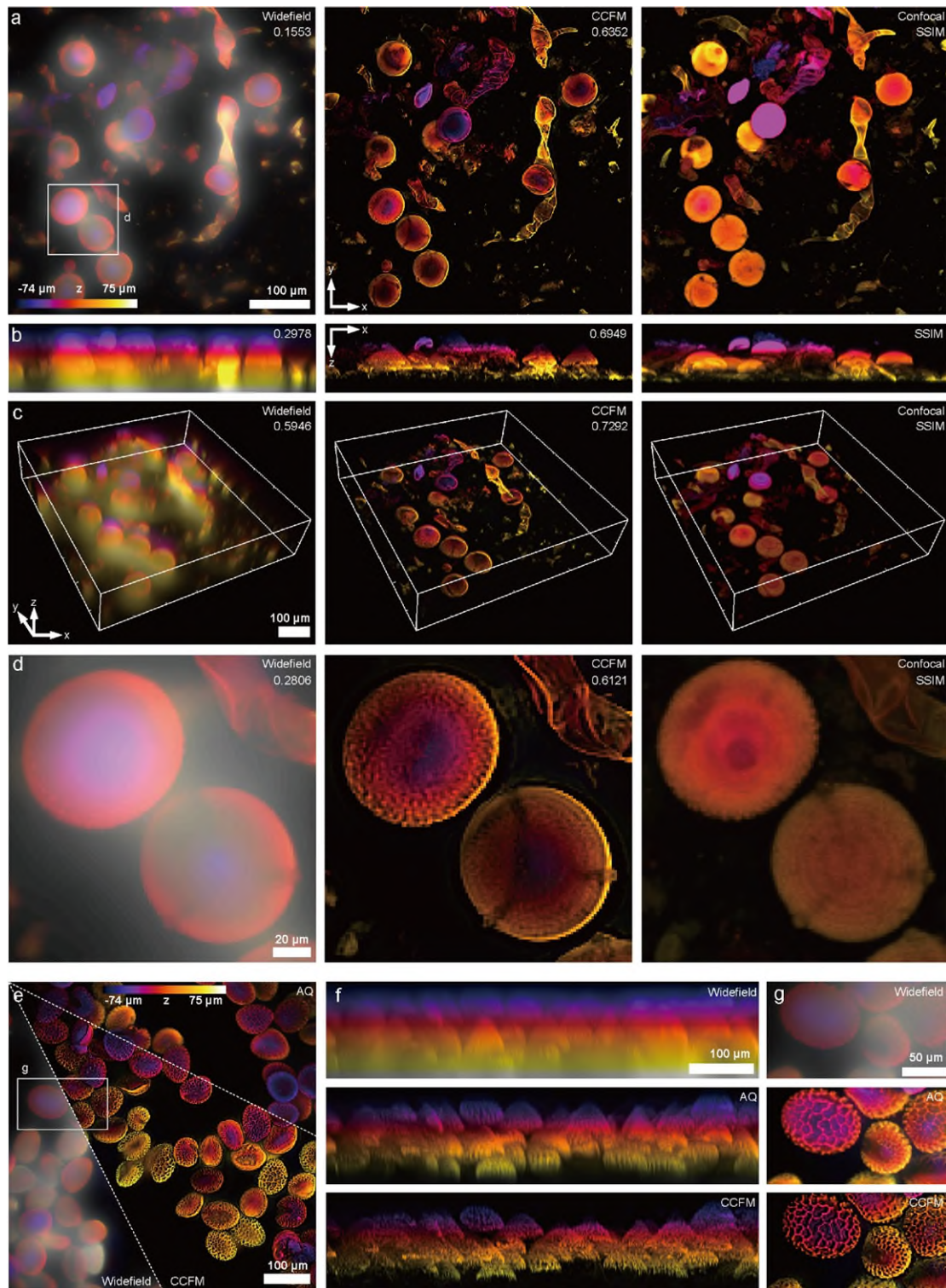
analysis, where 2D denotes the average of slice-wise SSIM values and 3D denotes the SSIM computed over the entire volume, showing the structural similarity index between CCFM reconstructions and the confocal reference as a function of iteration number. The curves indicate rapid improvement followed by stabilization of image quality. **i**, Structural similarity index of raw widefield, AQ, and CCFM relative to confocal for the slices shown in **b–g**. **j**, SSIM of raw widefield, AQ, and CCFM relative to confocal over depths from $-10\text{ }\mu\text{m}$ to $+15\text{ }\mu\text{m}$.

dimensional morphology of the trichome in close agreement with the confocal reference, indicating that the calibration captures the dominant composite transfer of the reference specimen under the current imaging condition. **b**, Axial slices of the calibrated TPSF over a depth range from $-30\text{ }\mu\text{m}$ to $+30\text{ }\mu\text{m}$, showing the depth-dependent evolution of the kernel. **c**, MIP of the calibrated TPSF on the left and the orthogonal y - z cross-section on the right, revealing pronounced asymmetry and broadening relative to the theoretical diffraction-limited PSF and reflecting the combined contributions of scattering-mediated redistribution and residual system response encoded in the TPSF. **d**, Three-dimensional volume rendering of the TPSF, visualizing the kernel morphology used for reconstruction of similar specimens.



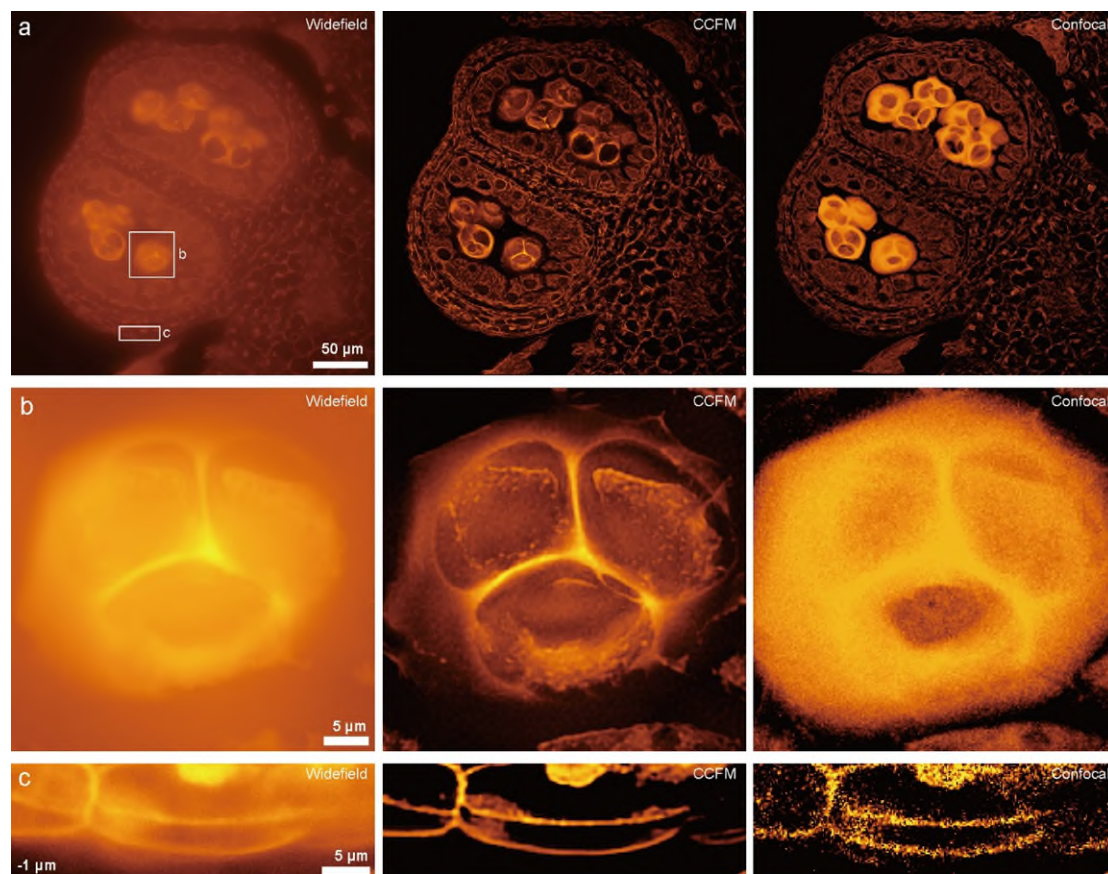
Extended Data Fig. 3 | Performance of CCFM in highly scattering specimens. **a**, MIP comparison of a butterfly leg specimen with a thickness of approximately 75 μm , imaged using a 20 $\times$, 0.75 NA objective. Panels show raw widefield, CCFM, AQ, and confocal images. CCFM suppresses the strong scattering haze intrinsic to chitinous tissue and recovers structures in closer agreement with the confocal reference. **b**, Enlarged view of the cuticle surface region marked in **a**. CCFM reconstruction recovers fine texture details and surface setae that are obscured by background signals in the widefield and AQ images. **c**, CDP of the claw region indicated in **a**, showing its three-dimensional topology. **d**, Orthogonal x - z cross-section along the dashed line in **c**. Owing to the lack of optical sectioning, the widefield image exhibits severe axial smearing, whereas AQ only partially constrains signal spread.

In contrast, CCFM achieves tighter axial confinement and stronger background suppression, approaching the optical sectioning observed in the confocal reference.

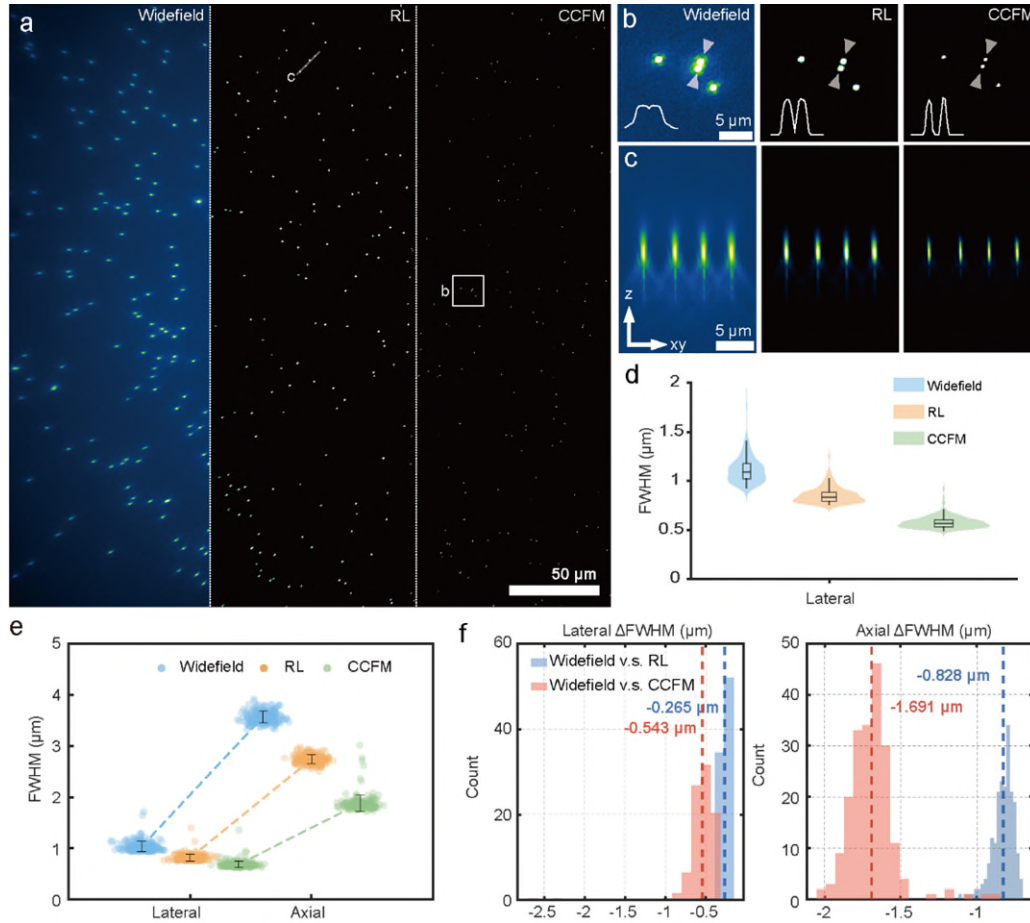


Extended Data Fig. 4 | Volumetric reconstruction of thick pollen grains. **a–d**, Validation on highly scattering *Cucurbita* pollen grains with a thickness of approximately 90 μm , imaged using a 20 $\times$, 0.75 NA objective. **a**, Color-coded depth projection comparing raw widefield, CCFM, and confocal images. Owing to severe volumetric scattering, the raw widefield image is dominated by defocused haze, whereas CCFM suppresses this background and recovers grain morphology consistent with the confocal reference. **b**, Corresponding orthogonal cross-sectional views for **a**. The widefield data lack optical sectioning, whereas CCFM achieves tighter axial confinement, reduces severe axial smearing, and reveals the hollow morphology of the grains observed in the confocal reference. **c**, Three-

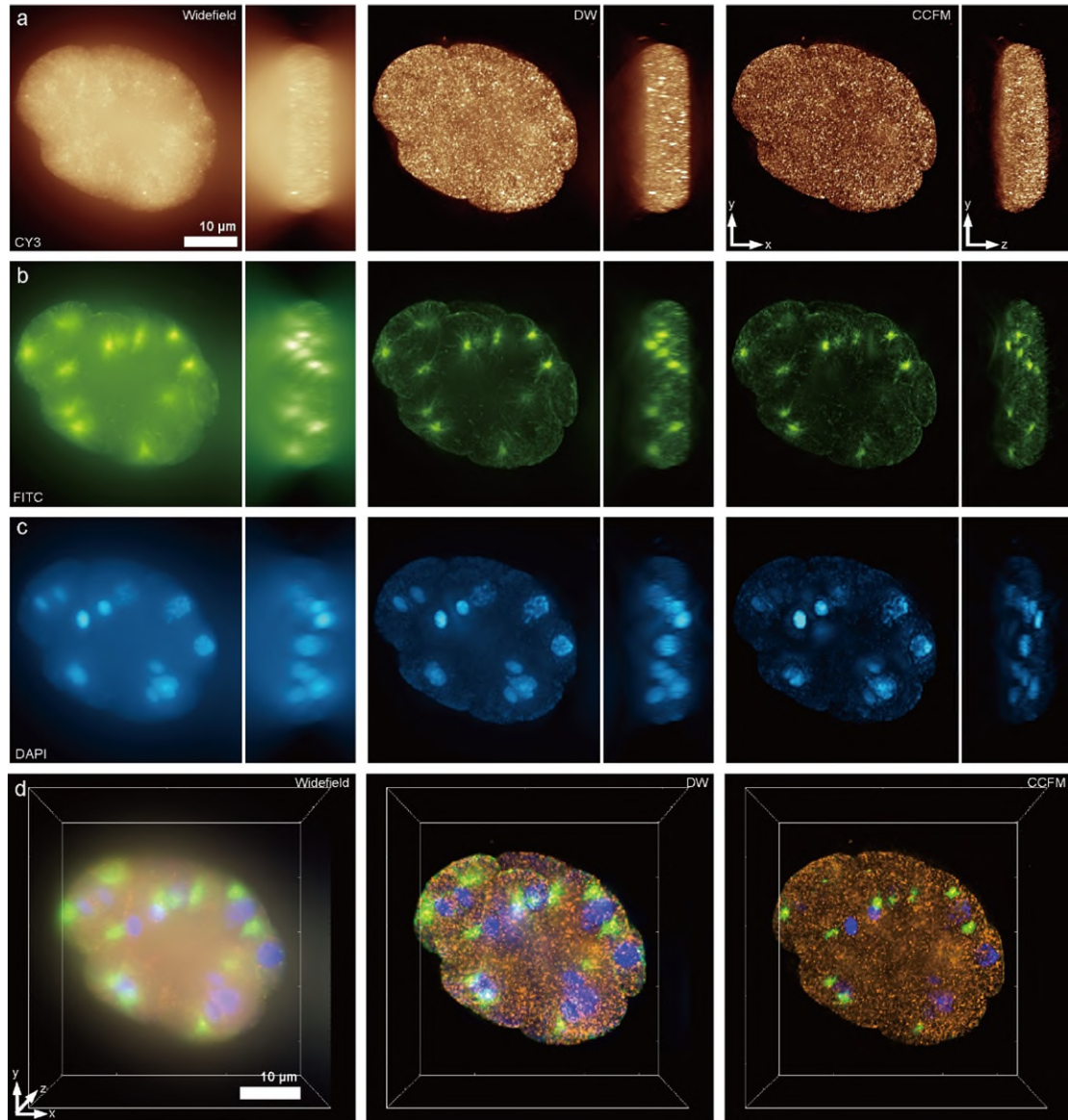
dimensional volume rendering. **d**, Enlarged view showing that fine surface details obscured by scattering in the widefield image are recovered more clearly by CCFM. **e–g**, Comparative imaging of highly scattering *Lilium* pollen grains with a thickness of approximately 100 μm . **e**, Large-field-of-view color-coded depth projection comparing raw widefield, CCFM, and AQ. **f**, Orthogonal view comparison of widefield on top, AQ in the middle, and CCFM on the bottom. The widefield image exhibits characteristic axial smearing, and although AQ partially reduces optical blur, it fails to suppress scattering-induced haze, leaving residual background and poorly defined axial boundaries. In contrast, CCFM achieves stronger background suppression and tighter axial confinement, revealing the folded morphology of the pollen grains more clearly. **g**, Enlarged view of the surface reticulate structure marked in **e**.



Extended Data Fig. 5 | Imaging of flower bud sections with a 40 $\times$, 0.9 NA objective. **a**, MIP comparison of raw widefield, CCFM and confocal for approximately 15 μm thick flower bud sections, showing effective removal of widefield blur by CCFM. **b,c**, Enlarged views of the internal cellular structures and the epidermal region marked in **a**. The confocal reference exhibits signal saturation and pronounced photon noise in high-intensity regions, whereas CCFM produces more balanced reconstructions with higher contrast and lower noise, resolving fine structural details such as the bilayer cell walls in **c**.



Extended Data Fig. 6 | Quantitative resolution characterization using fluorescent beads. a–c, Imaging of 100 nm fluorescent beads with a 40 $\times$, 0.9 NA objective. **a**, MIP comparison of widefield on the left, RL in the middle, and CCFM on the right. **b**, Enlarged lateral x–y view of the boxed region in **a**, with bead intensity profiles illustrating the improvement in bead resolvability. **c**, Orthogonal x–z cross-section along the line marked in **a**, comparing the axial elongation in widefield and RL with the tighter axial confinement achieved by CCFM. **d**, Violin plots of the lateral FWHM distributions for the three methods. **e**, Scatter plot showing the correlated decrease of axial and lateral FWHM across the bead population, highlighting the clustering of CCFM results toward the diffraction limit. **f**, Histograms of the FWHM reduction relative to widefield for RL in red and CCFM in green, with the left panel showing lateral ΔFWHM and the right panel showing axial ΔFWHM . Dashed lines indicate the mean improvement.



Extended Data Fig. 7 | TPSF based enhancement of a public dataset. **a–d**, Reprocessing of a publicly available *C. elegans* embryo dataset [17] acquired with a 100×, 1.4 NA objective. The dataset contains three channels: CY3 for protein puncta in **a**, FITC for microtubules in **b**, and DAPI for chromosomes in **c**. **a–c**, MIP views on the left and orthogonal y–z cross-sections on the right compare raw widefield, Deconvolf (DW) [22] and CCFM reprocessed results. Although DW improves lateral resolution, residual volumetric haze remains evident in the axial views, whereas CCFM suppresses this background more effectively and restores cleaner optical sectioning across all three spectral channels. **d**, Three-dimensional volume rendering of the merged tri-color dataset. Compared with widefield on the left and DW in the middle, CCFM on the right reveals the spatial organization of chromosomes, microtubules, and protein puncta with stronger background suppression, further demonstrating that CCFM can retrospectively enhance existing widefield datasets toward confocal-like contrast.

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