

Fast, three-dimensional, live-cell super-resolution imaging with multiplane structured illumination microscopy

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Supplementary Note. 1: 3D-MP-SIM forward model

3D-MP-SIM uses an illumination pattern identical to that of 3D-SIM, which is produced by the interference of three beams. Each beam can be represented in complex plane wave form $\mathbf{E}_j$ ($j=1\sim 3$) with the respective amplitude E_j , propagation vector $\mathbf{k}_j$, and phase φ_j , where j indicates the beam's order.

$$\mathbf{E}_j(\mathbf{r}) = E_j \exp(i(\mathbf{k}_j \cdot \mathbf{r} + \varphi_j)), j=1,2,3, \quad (1)$$

where $\mathbf{E}_1$ and $\mathbf{E}_2$ are symmetrical beam vectors with order 0 beam $\mathbf{E}_3$ as the center, and $\mathbf{E}_3$ travels along the optical axis. The amplitudes of beam vectors $\mathbf{E}_1$ and $\mathbf{E}_2$ are defined as aE (i.e., $aE = E_1 = E_2$, $a \in (1.2, 1.4)$); these are slightly greater than E_3 ($E_3=E$) to enhance the highest frequency information components¹. Then, a coherent superposition of these plane waves produces a total light intensity $I(\mathbf{r})$ as follows:

$$\begin{aligned} I(\mathbf{r}) &= (\mathbf{E}_1 + \mathbf{E}_2 + \mathbf{E}_3) \cdot (\mathbf{E}_1^* + \mathbf{E}_2^* + \mathbf{E}_3^*) \\ &= E^2 \{ 2a^2 + 1 + 2a^2 \cos[(\mathbf{k}_1 - \mathbf{k}_2) \cdot \mathbf{r} + \varphi_1 - \varphi_2] \\ &\quad + 2a \cos[(\mathbf{k}_1 - \mathbf{k}_3) \cdot \mathbf{r} + \varphi_1 - \varphi_3] + 2a \cos[(\mathbf{k}_2 - \mathbf{k}_3) \cdot \mathbf{r} + \varphi_2 - \varphi_3] \} \end{aligned} \quad (2)$$

According to the properties of coherent light, the moduli of the three propagation vectors are equal. Therefore, any subtraction of two plane-wave propagation vectors $\mathbf{k}_q - \mathbf{k}_j$ in Eq. (2) can be rewritten with the same transverse and vertical vectors $\mathbf{k}_{xy}$ and $\mathbf{k}_z$ in Cartesian coordinates through the following geometric relationships:

$$\mathbf{k}_1 - \mathbf{k}_2 = 2\mathbf{k}_{xy} \quad \mathbf{k}_1 - \mathbf{k}_3 = \mathbf{k}_{xy} + \mathbf{k}_z \quad \mathbf{k}_2 - \mathbf{k}_3 = -\mathbf{k}_{xy} + \mathbf{k}_z. \quad (3)$$

Therefore, Eq. (2) can be simplified as follows:

$$I(\mathbf{r}) = I_0 \left[1 + 2c_2 \cos(2\mathbf{k}_{xy} \cdot \mathbf{r}_{xy} + 2\varphi_{xy}) + 4c_1 \cos(\mathbf{k}_{xy} \cdot \mathbf{r}_{xy} + \varphi_{xy}) \cos(\mathbf{k}_z \cdot \mathbf{r}_z + \varphi_z) \right], \quad (4)$$

where $I_0 = \frac{E^2}{2a^2 + 1}$ is the mean intensity of the illumination, $c_1 = \frac{a}{2a^2 + 1}$ and $c_2 = \frac{a^2}{2a^2 + 1}$ are order 1 and order 2 modulation depths, respectively, and $\varphi_{xy} = (\varphi_1 - \varphi_2)/2$ and $\varphi_z = (\varphi_1 + \varphi_2 - 2\varphi_3)/2$ are the lateral and axial initial phases, respectively. The pattern $I(\mathbf{r})$ can be described as a sum of a finite number of components¹, with each component being separable into axial functions $I_m(z)$ and lateral functions $J_m(\mathbf{r}_{xy})$:

$$I(\mathbf{r}_{xy}, z) = \sum_m J_m(\mathbf{r}_{xy}) I_m(z), \quad (5)$$

where $\mathbf{r}_{xy}$ denotes the lateral coordinates (x, y) and z denotes the axial coordinate. Generally, the pattern illuminates the fluorescent sample, and the emitted fluorescence is then captured by the objective lens. This process can be described as follows:

$$\begin{aligned}
D(\mathbf{r}) &= \sum_m \left[S(\mathbf{r}) J_m(\mathbf{r}_{xy}) I_m(z) \right] \otimes H(\mathbf{r}) \\
&= \sum_m \int S(\mathbf{r}') J_m(\mathbf{r}'_{xy}) I_m(z') H(\mathbf{r} - \mathbf{r}') d\mathbf{r}',
\end{aligned} \tag{6}$$

where m ($m = \{-2, -1, 0, 1, 2\}$) is the order of the spectrum and $H(\mathbf{r})$ is the point spread function (PSF).

The difference between multiplane SIM and 3D-SIM is the detection path. For 3D-SIM imaging, each plane of the sample was moved to the focus of the objective lens, resulting in the countermovement of the sample $S(\mathbf{r})$ and the axial illumination pattern I_m . Therefore, axial illuminating functions I_m should not depend on the sample coordinate z' but on the objective-lens coordinate $z - z'$, which is the same as the coordinate of PSF (**Fig. 1c**):

$$\begin{aligned}
D(\mathbf{r}) &= \sum_m \int S(\mathbf{r}') J_m(\mathbf{r}'_{xy}) I_m(z - z') H(\mathbf{r} - \mathbf{r}') d\mathbf{r}' \\
&= \sum_m (S J_m) \otimes (H I_m).
\end{aligned} \tag{7}$$

For the multiplane SIM model, the sample of different depths were simultaneously imaged in distinct regions of the cameras and captured under a single illumination, resulting in alignment between the sample coordinate z' and the illumination pattern coordinate z' . Consequently, the axial frequency shifts are directly applied to the sample spectrum (**Fig. 1b**):

$$\begin{aligned}
D(r) &= \sum_m \int S(\mathbf{r}') J_m(\mathbf{r}'_{xy}) I_m(z') H(\mathbf{r} - \mathbf{r}') d\mathbf{r}' \\
&= \sum_m (S J_m I_m) \otimes H \\
&= [S(\mathbf{r}) I(\mathbf{r})] \otimes H(\mathbf{r})
\end{aligned} \tag{8}$$

Its Fourier spectrum can be represented as follows:

$$\tilde{D}(\mathbf{k}) = [\tilde{S}(\mathbf{k}) \otimes \tilde{I}_m(\mathbf{k})] O(\mathbf{k}), \tag{9}$$

where $O(\mathbf{k})$ is the OTF and $\mathbf{k}$ is the coordinate of the Fourier spectrum, and the superscript $\sim$ represents the Fourier spectrum of the data. Both order 1 and order 2 spectra of the sample are shifted to the order 0 spectral position, resulting in a total of seven spectral components filtered by the WF OTF.

Previous multiplane SIM methods²⁻⁴ usually used plane-by-plane reconstruction, which could not achieve axial super-resolution. For example, when applying a three-beam structured illumination pattern followed by multiplane detection^{3, 4}, the spectrum of the acquired 3D stack can be expressed as:

$$\begin{aligned}
\tilde{D}^l(\mathbf{k}) &= [\tilde{I}(\mathbf{k}) \otimes \tilde{S}(\mathbf{k})] O(\mathbf{k}) = c_0 O(\mathbf{k}) \tilde{S}(\mathbf{k}_{xy}, \mathbf{k}_z) + \\
&c_2 O(\mathbf{k}) \tilde{S}(\mathbf{k}_{xy} - 2\mathbf{p}_{xy}, \mathbf{k}_z) e^{2i\varphi_{xy} + (l-1)\frac{2\pi}{5}i} + c_2 O(\mathbf{k}) \tilde{S}(\mathbf{k}_{xy} + 2\mathbf{p}_{xy}, \mathbf{k}_z) e^{-2i\varphi_{xy} - (l-1)\frac{2\pi}{5}i} \\
&+ (c_1 O(\mathbf{k}) \tilde{S}(\mathbf{k}_{xy} - \mathbf{p}_{xy}, \mathbf{k}_z - \mathbf{p}_z) e^{i\varphi_z} + c_1 O(\mathbf{k}) \tilde{S}(\mathbf{k}_{xy} - \mathbf{p}_{xy}, \mathbf{k}_z + \mathbf{p}_z) e^{-i\varphi_z}) e^{+i\varphi_{xy} + (l-1)\frac{\pi}{5}i}, \\
&+ (c_1 O(\mathbf{k}) \tilde{S}(\mathbf{k}_{xy} + \mathbf{p}_{xy}, \mathbf{k}_z - \mathbf{p}_z) e^{i\varphi_z} + c_1 O(\mathbf{k}) \tilde{S}(\mathbf{k}_{xy} + \mathbf{p}_{xy}, \mathbf{k}_z + \mathbf{p}_z) e^{-i\varphi_z}) e^{-i\varphi_{xy} - (l-1)\frac{\pi}{5}i}
\end{aligned} \tag{10}$$

where $\tilde{D}^l(\mathbf{k})$ is the spectrum of the acquired images, l is the sequence number of the collected image

($l=1\sim 5$), I is the illumination pattern, S is spectrum of the sample, O is the optical transfer function, c is the modulation depths, and $\mathbf{p}_{xy}$ and $\mathbf{p}_z$ is the lateral and axial pattern vectors, respectively. φ_{xy} and φ_z is the lateral and axial initial phase of the illumination pattern, respectively. If the conventional 2D-SIM or 3D-SIM reconstruction algorithm is used to reconstruct the multiplane data, the five components at most can be derived as:

$$\begin{bmatrix} C_{m=-2} \\ C_{m=-1} \\ C_{m=0} \\ C_{m=+1} \\ C_{m=+2} \end{bmatrix} = \mathbf{M}_{5 \times 5}^{-1} \begin{bmatrix} \tilde{D}^1(\mathbf{k}) \\ \tilde{D}^2(\mathbf{k}) \\ \tilde{D}^3(\mathbf{k}) \\ \tilde{D}^4(\mathbf{k}) \\ \tilde{D}^5(\mathbf{k}) \end{bmatrix}, \quad (11)$$

where the $\mathbf{M}_{5 \times 5}$ is the separated matrix:

$$\mathbf{M}_{5 \times 5} = \begin{bmatrix} e^{-i2\varphi_0} & e^{-i\varphi_0} & 1 & e^{+i\varphi_0} & e^{+i2\varphi_0} \\ e^{-i2\varphi_1} & e^{-i\varphi_1} & 1 & e^{+i\varphi_1} & e^{+i2\varphi_1} \\ e^{-i2\varphi_2} & e^{-i\varphi_2} & 1 & e^{+i\varphi_2} & e^{+i2\varphi_2} \\ e^{-i2\varphi_3} & e^{-i\varphi_3} & 1 & e^{+i\varphi_3} & e^{+i2\varphi_3} \\ e^{-i2\varphi_4} & e^{-i\varphi_4} & 1 & e^{+i\varphi_4} & e^{+i2\varphi_4} \end{bmatrix}, \quad (12)$$

where $\varphi_0 = 0$, $\varphi_1 = 2\pi/5$, $\varphi_2 = 4\pi/5$, $\varphi_3 = 6\pi/5$, and $\varphi_4 = 8\pi/5$, which is in agreement with the $2\pi/5$ phase difference of two adjacent illumination patterns.

The C_m is the sample spectrum in Eq.(11), which consists of the lateral initial phase φ_{xy} , for order m ($m=0, \pm 1, \pm 2$). After removing the lateral initial phase φ_{xy} , C_m can be represented as:

$$\begin{aligned} C_0(\mathbf{k}) &= c_0 O(\mathbf{k}) S(\mathbf{k}_{xy}, \mathbf{k}_z) \\ C_1(\mathbf{k}) &= c_1 O(\mathbf{k}) S(\mathbf{k}_{xy} - \mathbf{p}_{xy}, \mathbf{k}_z - \mathbf{p}_z) e^{i\varphi_z} + c_1 O(\mathbf{k}) S(\mathbf{k}_{xy} - \mathbf{p}_{xy}, \mathbf{k}_z + \mathbf{p}_z) e^{-i\varphi_z} \\ C_{-1}(\mathbf{k}) &= c_1 O(\mathbf{k}) S(\mathbf{k}_{xy} + \mathbf{p}_{xy}, \mathbf{k}_z - \mathbf{p}_z) e^{i\varphi_z} + c_1 O(\mathbf{k}) S(\mathbf{k}_{xy} + \mathbf{p}_{xy}, \mathbf{k}_z + \mathbf{p}_z) e^{-i\varphi_z} \\ C_2(\mathbf{k}) &= c_2 O(\mathbf{k}) S(\mathbf{k}_{xy} - 2\mathbf{p}_{xy}, \mathbf{k}_z) \\ C_{-2}(\mathbf{k}) &= c_2 O(\mathbf{k}) S(\mathbf{k}_{xy} + 2\mathbf{p}_{xy}, \mathbf{k}_z) \end{aligned} \quad (13)$$

Either C_1 or C_{-1} contains two parts of axial super-resolution information and further separation can't be achieved. Therefore, after the lateral shift of each component by conventional 2D-SIM or 3D-SIM reconstruction pipeline:

$$\begin{aligned} C_1(\mathbf{k}) &= c_1 O(\mathbf{k}) S(\mathbf{k}_{xy}, \mathbf{k}_z - \mathbf{p}_z) e^{i\varphi_z} + c_1 O(\mathbf{k}) S(\mathbf{k}_{xy}, \mathbf{k}_z + \mathbf{p}_z) e^{-i\varphi_z} \\ C_{-1}(\mathbf{k}) &= c_1 O(\mathbf{k}) S(\mathbf{k}_{xy}, \mathbf{k}_z - \mathbf{p}_z) e^{i\varphi_z} + c_1 O(\mathbf{k}) S(\mathbf{k}_{xy}, \mathbf{k}_z + \mathbf{p}_z) e^{-i\varphi_z}, \end{aligned} \quad (14)$$

the upper and lower parts in C_1 and C_{-1} are still mixed and have not been restored to the correct axial position beyond the axial cut-off frequency of wide field microscopy. Therefore, the axial resolution remains equivalent to that of wide-field microscopy (**Extended Data Fig. 1b**).

For our proposed 3D-MP-SIM model, we introduced an axial phase shift, such as 0 and $\pi/2$, to separate seven spectral components, where the order 0 and order ± 2 components are similar to those in

3D-SIM, while the orders ± 1 spectrum are further separated into upper and lower parts (i.e., +1U, +1L, -1U, and -1L).

To achieve the 7 components separation, seven sets of raw data need to be acquired, including 5 images ($D^1 \sim D^5$) with a lateral phase shift of $2\pi/5$ and 2 images ($D^6 \sim D^7$) with a lateral phase shift of $4\pi/5$ after an axial phase shift of $\pi/2$:

$$\begin{bmatrix} \tilde{D}^1(\mathbf{k}) \\ \tilde{D}^2(\mathbf{k}) \\ \tilde{D}^3(\mathbf{k}) \\ \tilde{D}^4(\mathbf{k}) \\ \tilde{D}^5(\mathbf{k}) \\ \tilde{D}^6(\mathbf{k}) \\ \tilde{D}^7(\mathbf{k}) \end{bmatrix} = \mathbf{M}_{7 \times 7} \cdot \begin{bmatrix} C_{m=-2} \\ C_{m=-1L} \\ C_{m=-1U} \\ C_{m=0} \\ C_{m=+1U} \\ C_{m=+1L} \\ C_{m=+2} \end{bmatrix}, \quad (15)$$

where C_m is the sample spectrum filtered by $O(\mathbf{k})$ for order m . The 7×7 separation matrix $\mathbf{M}_{7 \times 7}$ can be represented as follows:

$$\mathbf{M}_{7 \times 7} = \begin{bmatrix} e^{0i} & e^{0i} & e^{0i} & 1 & e^{0i} & e^{0i} & e^{0i} \\ e^{-\frac{4\pi}{5}i} & e^{-\frac{2\pi}{5}i} & e^{-\frac{2\pi}{5}i} & 1 & e^{\frac{2\pi}{5}i} & e^{\frac{2\pi}{5}i} & e^{\frac{4\pi}{5}i} \\ e^{-\frac{8\pi}{5}i} & e^{-\frac{4\pi}{5}i} & e^{-\frac{4\pi}{5}i} & 1 & e^{\frac{4\pi}{5}i} & e^{\frac{4\pi}{5}i} & e^{\frac{8\pi}{5}i} \\ e^{-\frac{12\pi}{5}i} & e^{-\frac{6\pi}{5}i} & e^{-\frac{6\pi}{5}i} & 1 & e^{\frac{6\pi}{5}i} & e^{\frac{6\pi}{5}i} & e^{\frac{12\pi}{5}i} \\ e^{-\frac{16\pi}{5}i} & e^{-\frac{8\pi}{5}i} & e^{-\frac{8\pi}{5}i} & 1 & e^{\frac{8\pi}{5}i} & e^{\frac{8\pi}{5}i} & e^{\frac{16\pi}{5}i} \\ e^{0i} & e^{0i-\frac{\pi}{2}i} & e^{0i+\frac{\pi}{2}i} & 1 & e^{0i+\frac{\pi}{2}i} & e^{0i-\frac{\pi}{2}i} & e^{0i} \\ e^{-\frac{8\pi}{5}i} & e^{-\frac{4\pi}{5}i-\frac{\pi}{2}i} & e^{-\frac{4\pi}{5}i+\frac{\pi}{2}i} & 1 & e^{\frac{4\pi}{5}i+\frac{\pi}{2}i} & e^{\frac{4\pi}{5}i-\frac{\pi}{2}i} & e^{\frac{8\pi}{5}i} \end{bmatrix}. \quad (16)$$

However, the separation matrix $\mathbf{M}_{7 \times 7}$ has a greater condition number (Cond=5.2) than the classical 5×5 matrix (Cond=1) with a lateral phase difference of $2\pi/5$. The condition number is a concept that describes the numerical sensitivity to noise (**Supplementary Note. 6**). A higher condition number of 5.2 indicates that the $\mathbf{M}_{7 \times 7}$ matrix is more sensitive to small changes during separation, leading to vulnerability to noise during reconstruction (**Supplementary Fig. 8**). Instead, in our 3D-MP-SIM, two sets of 5 images with $2\pi/5$ lateral phase difference were captured before and after axial shifting by $\pi/2$, respectively. Then, employing the 10 raw images for separating the seven spectral components achieves a lower condition number of 1.4, resulting in high-performance reconstructions (**Supplementary Fig. 8**).

Supplementary Note. 2: Axial phase shift module

In SIM, the lateral movement of the grating displayed by the SLM corresponds to the translation of the lateral phase of the illumination pattern within the sample. Similarly, the translation of the SLM along the z-axis can also induce an axial phase shift. However, this method is insufficient for live-cell imaging and may result in alignment errors.

To address these challenges, an optical delay line, which consisted of a right-angle prism and a retroreflecting hollow roof prism mirror mounted on a piezo stage, was proposed. This module introduced an axial phase shift by altering the optical path length of the conjugate plane of the SLM relative to the sample, and the desired phase shift was uniformly applied in each conjugate plane. Theoretically, the axial phase shift φ_z could be achieved by the pattern movement s as follows:

$$s = \frac{\varphi_z \lambda}{2\pi(1 - \cos \theta)}, \quad (17)$$

where λ is the wavelength of the illumination light. The angle θ in the conjugate plane is 3° ; this angle is derived from the final excitation NA of 1.27 and the magnification of the two 4f systems from the conjugate plane of the SLM to the sample. Furthermore, the design of the optical delay line doubles the changes in the optical path difference relative to the changes in the stage movement. Therefore, to achieve an ideal phase shift of $\varphi_z = 0.5\pi$, the piezo stage only needs to translate $s/2 = 45 \mu\text{m}$.

Supplementary Note. 3: Measurement of the axial phase shift

To evaluate the performance of the axial phase shift module, an experiment utilizing two interfering beams was designed (**Supplementary Fig. 3**). While interference from the ± 1 diffraction orders produced conventional 2D SIM illumination, introducing an axial phase shift did not alter the initial lateral phase at the focal plane since the pattern remained constant along the optical axis (**Supplementary Fig. 3a**). In setups involving three-beam interference, obstructing one of the first-order beams resulted in a tilted 2D SIM pattern. The axial movement of this pattern generated structured illumination with a distinct initial lateral phase at the focal plane (**Supplementary Fig. 3b**). The lateral phase shift observed corresponded to the axial phase alternation used in 3D-MP-SIM (**Supplementary Fig. 3b**). The tilted pattern can be derived by $\mathbf{E}_1$ and $\mathbf{E}_3$:

$$\begin{aligned} I(\vec{r}) &= (\mathbf{E}_1 + \mathbf{E}_3) \cdot (\mathbf{E}_1^* + \mathbf{E}_3^*) \\ &= E^2 \left[a^2 + 1 + 2a \cos(\mathbf{k}_{xy} \cdot \mathbf{r}_{xy} + \mathbf{k}_z z + \varphi_1 - \varphi_3) \right] \end{aligned} \quad (18)$$

At specific z positions, such as the focal plane, the resulting pattern was sinusoidal, similar to 2D SIM. Evidently, a displacement Δz introduced an additional initial phase $\mathbf{k}_z \Delta z$, aligning with the desired axial phase in 3D-MP-SIM. Consequently, the axial phase shift introduced by the optical delay line was converted into a lateral phase shift, which was much easier to measure.

For practical implementation, 100-nm fluorescent beads affixed to the bottom of a Petri dish were used to conduct the 2D SIM experiment. The tilted structured illumination excited the sample in a sequence similar to that of conventional 2D SIM (**Supplementary Fig. 3b**). After capturing 9 raw images, the desired axial phase was introduced by the translation stage in the delay line, and a subsequent set of images was acquired. The resulting initial phase shift was measured to be approximately $\pi/2$ (1.63 ± 0.17 , mean $\pm$ s.d., $n = 100$). To further corroborate these results, a comparable experiment using conventional 2D SIM was performed to ensure that the optical configuration was correctly adjusted; this yielded a phase variation of approximately zero (0.00 ± 0.26 , mean $\pm$ s.d., $n = 150$).

Supplementary Note. 4: Reconstruction of 3D-MP-SIM data

The eight regions of the two cameras captured eight raw images within a thickness of 1.55 μm in the sample, and these regions needed to be registered before reconstruction. The registration parameters were derived from a specially designed pipeline involving the capture of the three-dimensional images of the beads by moving the z-piezo stage. Average intensity projections were then performed along the axial direction for each stack of the eight regions to produce eight two-dimensional images (**Supplementary Fig. 5**). By extracting scale-invariant feature transform features from the eight images and employing a feature-matching algorithm^a, the registration parameters could be calculated and applied to align the cell samples. Notably, the z-piezo stage is used only during the calibration process to collect the three-dimensional beads data for determining the registration parameters, while it remains stationary during 3D-MP-SIM imaging.

The reconstruction pipeline for 3D-MP-SIM involves data preprocessing (i.e., background removal⁵, normalization, and axial padding), spectral separation, spectral shifting, and Wiener filtering, similar to 3D-SIM. As discussed in **Supplementary Note. 1**, the difference between 3D-MP-SIM and 3D-SIM lies in that the former consists of seven spectral components mixed at the order 0 position; thus, the seven components (0, +1U, +1L, -1U, -1L, +2, and -2) need to be separated. The most straightforward way to acquire these seven individual spectra was to use a 7×7 matrix for separation in each direction, but this approach has a condition number of 5.2 (**Supplementary Note. 6**). Therefore, a two-step separation process with a condition number of 1.4 was proposed to enhance robustness.

In the first step, similar to 3D-SIM, five raw images (i.e., $D^1 \sim D^5$) were captured with a phase shift of $2\pi/5$ in each of the three directions d ($d=1,2,3$), for a total of 15 frames. Using the ISP and two cameras, eight different depths could be simultaneously captured at each of the phases and directions. All 120 images were stacked in sequence according to their phases φ , directions d , and depths into a single stack. Using a separation process similar to that used for 3D-SIM, five three-dimensional spectra (C_m , $m = \pm 2, \pm 1, 0$) corresponding to order m were preliminarily extracted in each direction from the 5 three-dimensional images ($D^1 \sim D^5$) with a lateral phase difference of $2\pi/5$:

$$\begin{bmatrix} \tilde{D}_{\Delta_z=0}^1(\mathbf{k}) \\ \tilde{D}_{\Delta_z=0}^2(\mathbf{k}) \\ \tilde{D}_{\Delta_z=0}^3(\mathbf{k}) \\ \tilde{D}_{\Delta_z=0}^4(\mathbf{k}) \\ \tilde{D}_{\Delta_z=0}^5(\mathbf{k}) \end{bmatrix} = \mathbf{M}_{5 \times 5} \cdot \begin{bmatrix} C_{m=-2, \Delta_z=0} \\ C_{m=-1, \Delta_z=0} \\ C_{m=0, \Delta_z=0} \\ C_{m=+1, \Delta_z=0} \\ C_{m=+2, \Delta_z=0} \end{bmatrix}. \quad (19)$$

where the $\tilde{D}_{\Delta_z=0}(\mathbf{k})$ is the spectrum of the captured raw images according to the axial phase shift Δ_z ,

^a https://docs.opencv.org/4.x/d7/d60/classcv_1_1SIFT.html

C_{m,Δ_z} is the separated three-dimensional spectra according to the order m and the $\mathbf{M}_{5 \times 5}$ is the separated matrix:

$$\mathbf{M}_{5 \times 5} = \begin{bmatrix} e^{-i2\varphi_0} & e^{-i\varphi_0} & 1 & e^{+i\varphi_0} & e^{+i2\varphi_0} \\ e^{-i2\varphi_1} & e^{-i\varphi_1} & 1 & e^{+i\varphi_1} & e^{+i2\varphi_1} \\ e^{-i2\varphi_2} & e^{-i\varphi_2} & 1 & e^{+i\varphi_2} & e^{+i2\varphi_2} \\ e^{-i2\varphi_3} & e^{-i\varphi_3} & 1 & e^{+i\varphi_3} & e^{+i2\varphi_3} \\ e^{-i2\varphi_4} & e^{-i\varphi_4} & 1 & e^{+i\varphi_4} & e^{+i2\varphi_4} \end{bmatrix}, \quad (20)$$

where $\varphi_0 = 0$, $\varphi_1 = 2\pi/5$, $\varphi_2 = 4\pi/5$, $\varphi_3 = 6\pi/5$, and $\varphi_4 = 8\pi/5$, which is in agreement with the $2\pi/5$ phase difference of two adjacent illumination patterns.

In Eq. (19), either $C_{m=-1,\Delta_z=0}$ or $C_{m=+1,\Delta_z=0}$ mixes both the upper and lower parts of the corresponding order m spectra, which requires further separation. To this end, an optical delay line was utilized to generate an axial phase shift during imaging. After altering the axial phase with $\pi/2$ (i.e., $\Delta_z = \pi/2$), each of the eight regions could capture another set of 15 raw images in five phases and three directions. Using a similar separation method:

$$\begin{bmatrix} \tilde{D}_{\Delta_z=\pi/2}^1(\mathbf{k}) \\ \tilde{D}_{\Delta_z=\pi/2}^2(\mathbf{k}) \\ \tilde{D}_{\Delta_z=\pi/2}^3(\mathbf{k}) \\ \tilde{D}_{\Delta_z=\pi/2}^4(\mathbf{k}) \\ \tilde{D}_{\Delta_z=\pi/2}^5(\mathbf{k}) \end{bmatrix} = \mathbf{M}_{5 \times 5} \cdot \begin{bmatrix} C_{m=-2,\Delta_z=\pi/2} \\ C_{m=-1,\Delta_z=\pi/2} \\ C_{m=0,\Delta_z=\pi/2} \\ C_{m=+1,\Delta_z=\pi/2} \\ C_{m=+2,\Delta_z=\pi/2} \end{bmatrix}. \quad (21)$$

Eq. (21) is similar to the Eq.(19) except the axial phase $\pi/2$. From the Eq. (21), order -1 component $C_{m=-1,\Delta_z=\pi/2}$ and order $+1$ component $C_{m=+1,\Delta_z=\pi/2}$ were obtained corresponding to the axial phase shift $\Delta_z = \pi/2$.

In the second step, we further separated the lower part $C_{m=-1,m_z=-1}$ and upper part $C_{m=-1,m_z=+1}$ of order -1 by employing a 2×2 separation matrix $\mathbf{M}_{2 \times 2}$:

$$\begin{bmatrix} C_{m=-1,\Delta_z=0} \\ C_{m=-1,\Delta_z=\pi/2} \end{bmatrix} = \mathbf{M}_{2 \times 2} \cdot \begin{bmatrix} C_{m=-1,m_z=-1} \\ C_{m=-1,m_z=+1} \end{bmatrix} = \begin{bmatrix} e^{-0i} & e^{+0i} \\ e^{-\frac{\pi}{2}i} & e^{+\frac{\pi}{2}i} \end{bmatrix} \begin{bmatrix} C_{m=-1,m_z=-1} \\ C_{m=-1,m_z=+1} \end{bmatrix}, \quad (22)$$

where $C_{m=-1,\Delta_z=0}$ and $C_{m=-1,\Delta_z=\pi/2}$ are overlapped order -1 components, which are already obtained from Eq.(19) and Eq.(21). The lower part $C_{m=-1,m_z=-1}$ and the upper part $C_{m=-1,m_z=+1}$ of order -1 separated from Eq. (22) can be represented as following:

$$C_{m,m_z} = c_{m,m_z} e^{i(m\varphi_{xy} + m_z\varphi_z)} C(\mathbf{k} - m\mathbf{p}_{xy}, \mathbf{k} - m_z\mathbf{p}_z) O(\mathbf{k}), \quad (23)$$

where m_z is the axial order, c_{m,m_z} is the modulation depth, φ_{xy} is the lateral initial phase, and φ_z is the axial initial phase. Similarly, for order +1 spectra, the lower part $C_{m=+1,m_z=-1}$ and the upper part $C_{m=+1,m_z=+1}$ can also be extracted using the same method of Eq. (22) and represented by Eq. (23). In practice, the axial phase shift Δ_z measured from the system is 0.52π (**Supplementary Note. 3, Supplementary Fig. 3**), which would substitute for $\pi/2$ in the separation matrix of Eq. (22) when reconstructing the actual data.

Besides the order ± 1 spectra, the order 0 and order ± 2 spectra can also be represented by Eq. (23). For example, although the order 0 spectra were extracted twice at different axial phase shifts Δ_z of 0 and $\pi/2$ (i.e., $C_{m=0,\Delta_z=0}$ and $C_{m=0,\Delta_z=\pi/2}$) from Eq. (19) and Eq. (21), these two replicas are theoretically equivalent and can be denoted as the same symbol $C_{m=0,m_z=0}$. Similarly, the order ± 2 spectra (i.e., $C_{m=\pm 2,\Delta_z=0}$ and $C_{m=\pm 2,\Delta_z=\pi/2}$) obtained from Eq. (19) and Eq. (21) were also extracted twice and can be denoted as $C_{m=\pm 2,m_z=0}$, where $m_z=0$ represents that the order 0 and order ± 2 spectra only have lateral spectral movement. Under the symbol definition, all seven spectra, including the order 0 spectra $C_{m=0,m_z=0}$, the order ± 1 spectra $C_{m=\pm 1,m_z=\pm 1}$ and order ± 2 spectra $C_{m=\pm 2,m_z=0}$, could be exactly represented by the universal expression of Eq. (23).

After separating seven components C_{m,m_z} , the lateral pattern vector $\mathbf{p}_{xy}$ and axial pattern vector $\mathbf{p}_z$ need to be estimated. The calculation method for $\mathbf{p}_{xy}$ is the same as that for 3D-SIM, but the axial pattern vector $\mathbf{p}_z$ is extra for 3D-MP-SIM, which can be obtained through theoretical computation or three-dimensional cross-correlation between order 0 spectra $C_{m=0,m_z=0}$ and the upper part of order +1 spectra $C_{m=+1,m_z=+1}$. Then, with the estimated vectors ($m\mathbf{p}_{xy}$, $m_z\mathbf{p}_z$), the high-order (i.e., $m=\pm 1, \pm 2$) spectra could be shifted to their original positions.

Similar to that in 3D-SIM, to estimate the lateral initial phase φ_{xy} , axial initial phase φ_z and modulation depth c_{m,m_z} of the high-order components C_{m,m_z} ($m=\pm 1, \pm 2$), a complex linear regression was employed within the overlapped regions of the zero- and high-order components. Finally, the initial phases and modulation depth were removed from the high-order spectra, and all spectra were merged through a generalized Wiener filter:

$$\tilde{S}(k) = \frac{\sum_{m,m_z} C_{m,m_z}(\mathbf{k} + m\mathbf{p}_{xy}, \mathbf{k} + m_z\mathbf{p}_z) O_{m,m_z}(\mathbf{k} + m\mathbf{p}_{xy}, \mathbf{k} + m_z\mathbf{p}_z)}{\sum_{m,m_z} |O_{m,m_z}(\mathbf{k} + m\mathbf{p}_{xy}, \mathbf{k} + m_z\mathbf{p}_z)|^2 + w^2} A(\mathbf{k}). \quad (24)$$

As mentioned earlier, the order 0 and ± 2 spectra C_{m,m_z} were extracted twice at axial phase shifts of 0 and $\pi/2$. These two replicas C_{m,m_z} were employed in the Wiener filter to enhance the signal-to-noise

ratio. Correspondingly, the denominator of the Wiener filter also doubled the OTFs corresponding to the order 0 and ± 2 .

Supplementary Note. 5: 3D-MP-SIM simulations

To validate the three-dimensional super-resolution imaging capabilities of 3D-MP-SIM, the following simulation experiments were conducted:

1. Generation of the Ground Truth

CS-3D simulation data with axial distribution were employed; this consisted of 512×512 pixels in the lateral direction with a $32.5 \text{ nm} \times 32.5 \text{ nm}$ pixel size and 64 planes in the axial direction with 24 nm spacing. Adjacent dots were inserted with a 97.5 nm lateral spacing or 292.5 nm axial spacing to assist in measuring the resolution of the reconstructed images.

2. Generation of the illumination pattern and PSF

To simulate the home-built 3D-MP-SIM, the illumination numerical aperture (NA) of the objective lens was set to 1.27. Based on Eq.(2), the three beams interfere with each other and form a three-dimensional structured illumination pattern. In addition to generating 15 illumination patterns similar to those in 3D-SIM (5 phases $\times$ 3 directions), 3D-MP-SIM generated another 15 illumination patterns with an axial phase shift of 0.52π to simulate the axial phase shift. The pixel size and pixel number of each illumination pattern were matched with those of the ground truth data. The three-dimensional PSF was generated using the open-source code^b based on the Hanser model⁶, with a detection NA of 1.333, a medium refractive index of 1.519, and a wavelength of 525 nm.

3. Generation of the 3D-SIM and 3D-MP-SIM raw images

For 3D-SIM, to simulate the axial movement for the capture of the different-depth samples, the ground truth data were axially moved 16 times, and each time was at an interval of 97.5 nm. At each z step, the ground truth data were multiplied by the illumination patterns and then convolved with the 3D PSF. Subsequently, the image of the convolution result was gathered and then laterally downsampled by a factor of 2 to obtain a raw image at each of the phases, directions, and focal planes.

For 3D-MP-SIM, the ground truth data were multiplied by the structured illumination pattern only once in each phase and direction, followed by convolution with the PSF. To simulate the pipeline, the illumination pattern remained stationary, and the focal planes of the eight regions were placed at eight planes in ground truth data with an axial interval of 195.0 nm, resulting in 8 convolution results. Then, the 8 images were laterally downsampled by a factor of 2 to obtain 8 raw images at each of the phases and directions.

Considering CS-3D data consisted of 64 planes in the axial direction with 24 nm spacing, 3D-SIM had a fourfold axial down sampling rate, while 3D-MP-SIM have doubled this to eightfold; this resulted in 16 planes of raw images with axial intervals of 97.5 nm and 8 planes of raw images with axial intervals of 195.0 nm, respectively.

^b <https://github.com/david-hoffman/pyotf>

4. Reconstruction and comparison

Finally, custom-developed algorithms were employed to reconstruct 3D-SIM and 3D-MP-SIM images, and their super-resolution counterparts were obtained (**Supplementary Fig. 6**). These results showed similar lateral and axial resolutions, as well as similar point and line details, demonstrating the potential of 3D-MP-SIM to achieve three-dimensional super-resolution imaging comparable to that of 3D-SIM.

Supplementary Note. 6: Calculation of condition numbers

The separation process in the reconstruction of the SIM, as illustrated in Eq.(15), can be treated as to solve the equation $\mathbf{b}=\mathbf{A}\mathbf{x}$. Here, $\mathbf{b}$ represents the known data, i.e., the raw image collected by SIM, $\mathbf{A}$ is the separation matrix, and $\mathbf{x}$ comprises the seven spectral components that need to be determined. The condition number of $\mathbf{A}$ indicates how sensitive the solution $\mathbf{x}$ is to changes or disturbances in $\mathbf{b}$.

For the seven-dimension separation matrix $\mathbf{M}_{7 \times 7}$ in Eq.(16), the condition number can be directly calculated by the definition formula, resulting in a value of 5.2.

To calculate the condition number of the two-step separation method proposed in **Supplementary Note. 4**, we needed to represent the two-step separation process as a single matrix equation:

$$\mathbf{M}_{10 \times 10}^{-1} \begin{bmatrix} \tilde{D}_{\Delta_z=0}^1(\mathbf{k}) \\ \tilde{D}_{\Delta_z=0}^2(\mathbf{k}) \\ \tilde{D}_{\Delta_z=0}^3(\mathbf{k}) \\ \tilde{D}_{\Delta_z=0}^4(\mathbf{k}) \\ \tilde{D}_{\Delta_z=0}^5(\mathbf{k}) \\ \tilde{D}_{\Delta_z=\pi/2}^1(\mathbf{k}) \\ \tilde{D}_{\Delta_z=\pi/2}^2(\mathbf{k}) \\ \tilde{D}_{\Delta_z=\pi/2}^3(\mathbf{k}) \\ \tilde{D}_{\Delta_z=\pi/2}^4(\mathbf{k}) \\ \tilde{D}_{\Delta_z=\pi/2}^5(\mathbf{k}) \end{bmatrix} = \begin{bmatrix} C_{m=-2, \Delta_z=0} \\ C_{m=-1, m_z=-1} \\ C_{m=0, \Delta_z=0} \\ C_{m=+1, m_z=-1} \\ C_{m=+2, \Delta_z=0} \\ C_{m=-2, \Delta_z=\pi/2} \\ C_{m=-1, m_z=+1} \\ C_{m=0, \Delta_z=\pi/2} \\ C_{m=+1, m_z=+1} \\ C_{m=+2, \Delta_z=\pi/2} \end{bmatrix}, \quad (25)$$

where the $\mathbf{M}_{10 \times 10}^{-1}$ is the inverse of separation matrix for the two-step separation processes and can be represented as:

$$\mathbf{M}_{10 \times 10}^{-1} = \begin{bmatrix} 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0.5 & 0 & 0 & 0 & 0 & +0.5i & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0.5 & 0 & 0 & 0 & 0 & +0.5i & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\ 0 & 0.5 & 0 & 0 & 0 & 0 & -0.5i & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0.5 & 0 & 0 & 0 & 0 & -0.5i & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 \end{bmatrix} \begin{bmatrix} \mathbf{M}_{5 \times 5}^{-1} & \mathbf{0}_{5 \times 5} \\ \mathbf{0}_{5 \times 5} & \mathbf{M}_{5 \times 5}^{-1} \end{bmatrix}, \quad (26)$$

where $\mathbf{M}_{5 \times 5}^{-1}$ is the inverse of $\mathbf{M}_{5 \times 5}$ in Eq.(20) to achieve the first-step separation. In the 2nd row and 7th

row of Eq.(26), the non-zero number can compose a matrix $\begin{bmatrix} 0.5 & +0.5i \\ 0.5 & -0.5i \end{bmatrix}$, which is calculated from the

inverse of matrix $\mathbf{M}_{2 \times 2}$ in Eq.(22), to achieve the second-step separation. The matrix $\mathbf{M}_{10 \times 10}^{-1}$, which is

obtained by multiplying these two matrices, can be used to convert the captured raw image to separated spectra by Eq.(25) .

Finally, according to the definition of the condition number, the condition number of the two-step separation processes, i.e., matrix $\mathbf{M}_{10 \times 10}$, which is the inverse of the matrix $\mathbf{M}_{10 \times 10}^{-1}$, can be computed as 1.4.

It should be noted that the two-step separation method written in **Supplementary Note. 4** can be completed in one step according to Eq.(25). The two-step representation in **Supplementary Note. 4** is provided to facilitate a detailed understanding of the separation process.

Supplementary Note. 7: Imaging thick samples using 3D-MP-SIM by axially moving the sample.

To image the thicker samples, we incorporated movement of the sample with a piezo stage into the current imaging process. This allows for the sequential imaging of multiple ‘eight-plane’ units (**Extended Data Fig. 3**), which can be concatenated into a volume for reconstruction. Implementing this strategy requires that the same region of the sample is illuminated by the same pattern before and after movements (indicated by two blue arrowheads in **Extended Data Fig. 3**). To this end, the distance of the axial movement z_0 should be an integer multiple of both the illumination pattern's axial period and the distance between two planes.

Consider the scenario of imaging a sample that exceeds a thickness of 1.55 μm , hypothetically represented as:

$$D(\mathbf{r}) = \int_0^{z+z_0} S(\mathbf{r}') I(\mathbf{r}') H(\mathbf{r} - \mathbf{r}') d\mathbf{r}', \quad (27)$$

where $D(\mathbf{r})$ is the hypothetically acquired stack for the entire thick sample, z is the depth that could be captured with 8 planes, i.e., 1.55 μm . The upper limit of integral $z+z_0$ represents the depth beyond 1.55 μm and $\mathbf{r}'$ is the coordinate system relative to the objective lens.

However, during practical imaging with 3D-MP-SIM, only 1.55 μm of the sample, composed of 8 planes (red solid box in left panel of **Extended Data Fig. 3**), can be captured at a time, denoted as $D_1(\mathbf{r})$:

$$D_1(\mathbf{r}) = \int_0^z S(\mathbf{r}') I(\mathbf{r}') H(\mathbf{r} - \mathbf{r}') d\mathbf{r}'. \quad (28)$$

To image the sample with a depth beyond 1.55 μm (blue dashed boxes in left panel of **Extended Data Fig. 3**), a new eight-plane stack $D_2(\mathbf{r})$ (blue solid box in middle panel of **Extended Data Fig. 3**) is acquired after axially shifting the sample by z_0 :

$$D_2(\mathbf{r}) = \int_0^z S(\mathbf{r}' + z_0) I(\mathbf{r}') H(\mathbf{r} - \mathbf{r}' - z_0) d\mathbf{r}'. \quad (29)$$

The integration interval remains $(0, z)$ because the coordinate system $\mathbf{r}'$ is based on the stationary objective lens and illumination pattern (**Extended Data Fig. 3**). If the movement z_0 is an integer multiple of the axial period of the illumination pattern I , the pattern $I(\mathbf{r}')$ in Eq.(29) is the same as $I(\mathbf{r}' + z_0)$:

$$I(\mathbf{r}') = I(\mathbf{r}' + z_0). \quad (30)$$

Substituting the Eq.(30) into the Eq.(29) can obtain:

$$D_2(\mathbf{r}) = \int_0^z S(\mathbf{r}' + z_0) I(\mathbf{r}' + z_0) H(\mathbf{r} - \mathbf{r}' - z_0) d\mathbf{r}'. \quad (31)$$

By changing the variable $\mathbf{r}' + z_0$ to $\mathbf{r}''$, we obtained:

$$D_2(\mathbf{r}) = \int_{z_0}^{z+z_0} S(\mathbf{r}'') I(\mathbf{r}'') H(\mathbf{r} - \mathbf{r}'') d\mathbf{r}''. \quad (32)$$

The raw image $D_2(\mathbf{r})$, which is captured after shifting the sample with z_0 distance, is equivalent to the hypothetical raw image $D(\mathbf{r})$ acquired without shifting in Eq.(27), among the depth z_0 and $z + z_0$. This means collecting data (blue solid box in middle panel of **Extended Data Fig. 3**) after axial movement

z_0 is completely equivalent to directly collecting data before movement (blue dashed box in left panel of **Extended Data Fig. 3**), if the axial movement distance z_0 is an integer multiple of the illumination pattern's axial period. Therefore, we can simply axially concatenate the practically captured $D_1(\mathbf{r})$ and $D_2(\mathbf{r})$ to one stack and reconstruct it with the conventional 3D-MP-SIM pipeline.

Additionally, to maintain an equidistance of adjacent plane after concatenating multiple 'eight-plane' units, the axial movement z_0 should also satisfy:

$$z_0 = k \cdot \text{Lspace} , \quad (33)$$

where k is an integer between 1 and 8, and Lspace represents the distance between two planes, which is 193.8 nm in our system.

By considering these constraints, we set the axial movement z_0 to 1.356 μm , corresponding to seven times the plane spacing, and twice the axial period P of the illumination pattern, which is 678 nm calculated from the following formula:

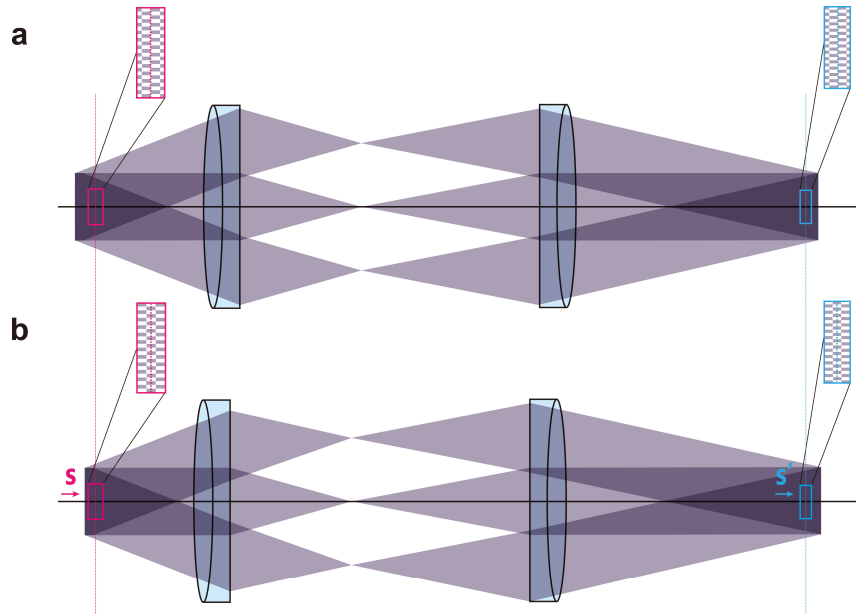
$$P = \frac{\lambda}{n \left(1 - \cos \left(\arcsin \left(\frac{\text{NA}_{\text{exc}}}{n} \right) \right) \right)} , \quad (34)$$

where P is axial period along of the illumination pattern, λ is the excitation wavelength as 488 nm, n is the refractive index of water as 1.333, and NA_{exc} is the excitation NA of 1.184 for aqueous-mounted samples in our 3D-MP-SIM.

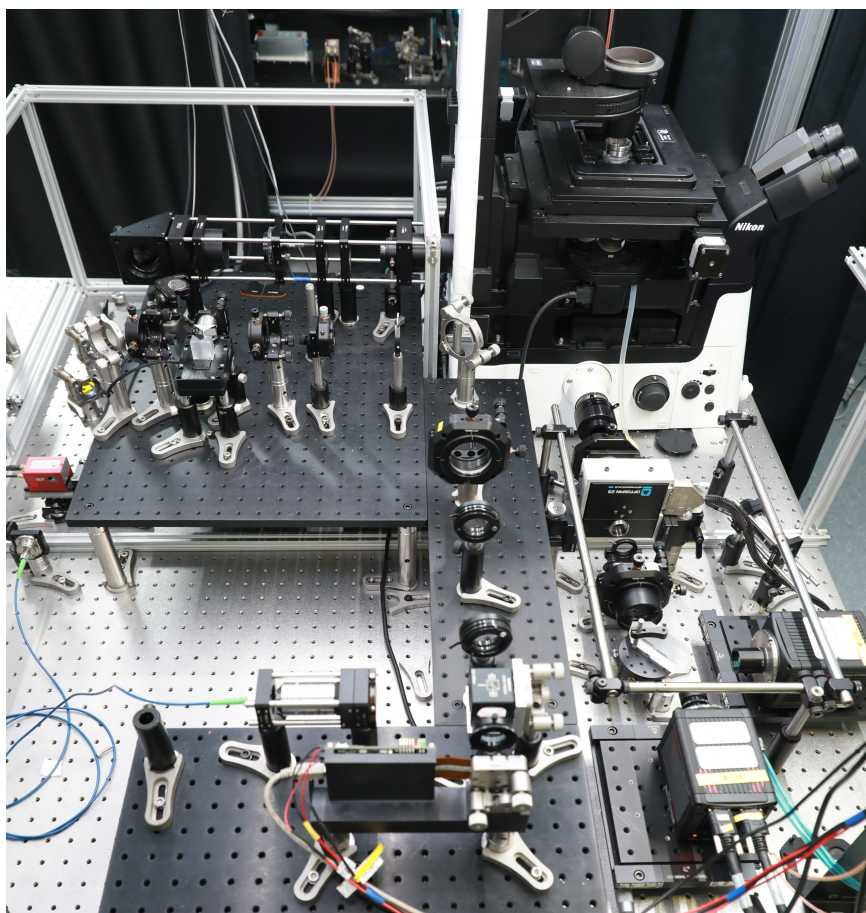
With the 1.356 μm of axial movement, the 1st plane of the second stack D_2 aligns with the 8th plane of the first stack D_1 , allowing us to discard one of them. The 2nd to 8th planes of the second stack D_2 can then be directly concatenated axially after the first eight-plane stack D_1 . The concatenated stack can subsequently be directly reconstructed using our previous proposed 3D-MP-SIM algorithm. This procedure can be readily extended to include three or more additional axially shifted stacks.

References

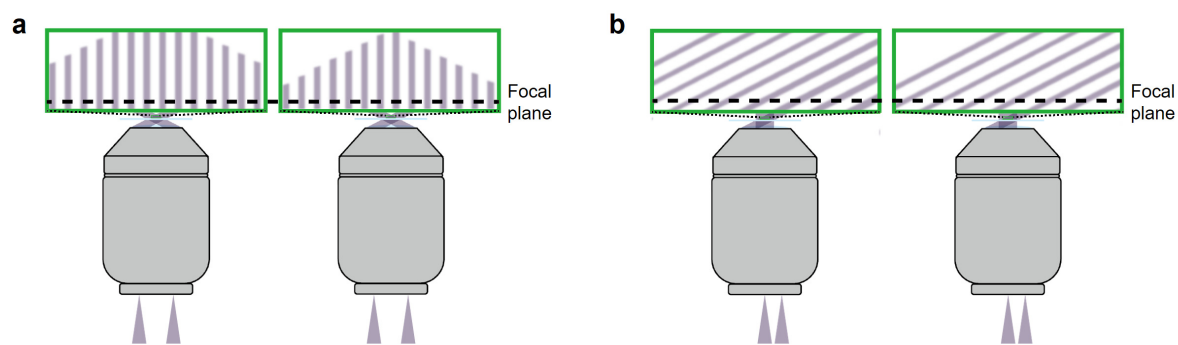
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Supplementary Fig. 1: Axial phase shift module. **a**, Interference pattern at the conjugate plane of the SLM and the corresponding interference pattern at the sample plane relayed by the 4f system. **b**, Relay of the interference pattern in the SLM conjugate plane inducing the corresponding phase shift in the sample plane.

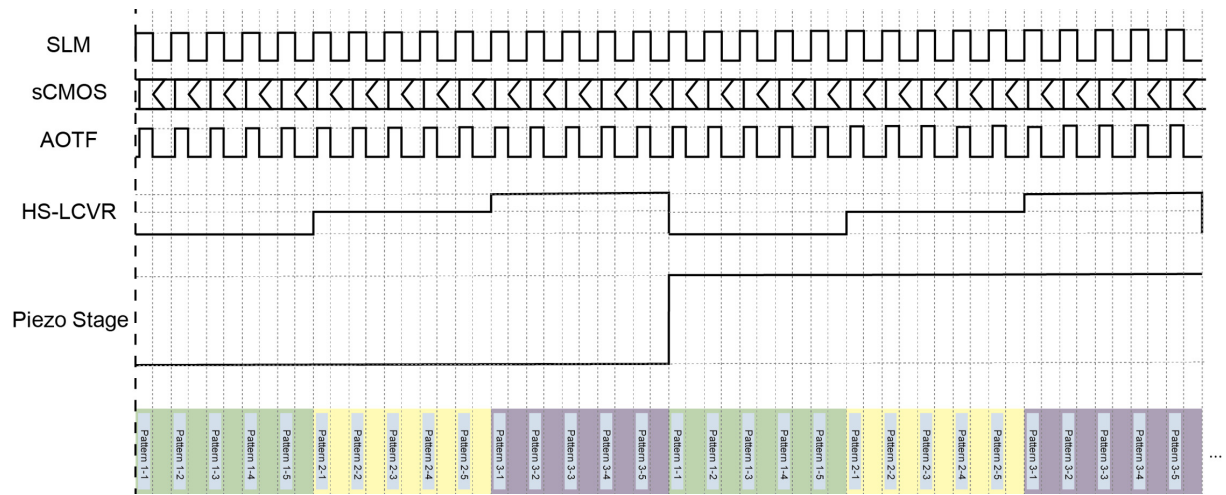


Supplementary Fig. 2: Photograph of 3D-MP-SIM microscope.

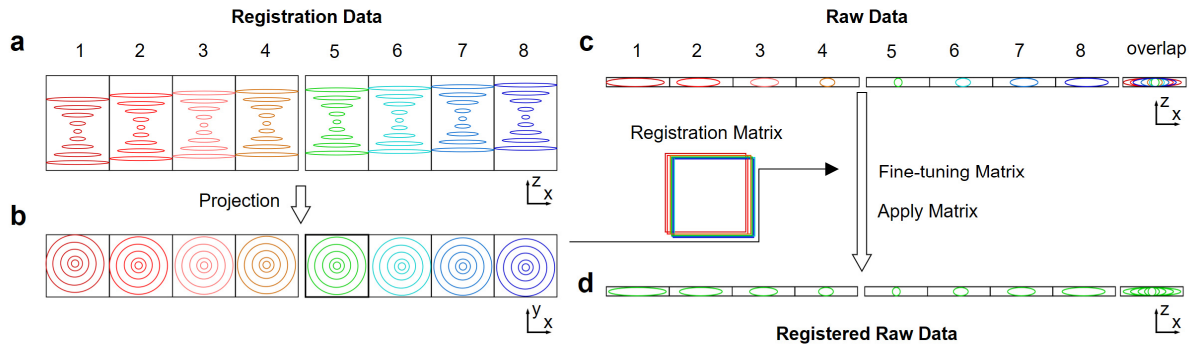


Supplementary Fig. 3: Measurement of the axial phase shift generated by the optical delay line.

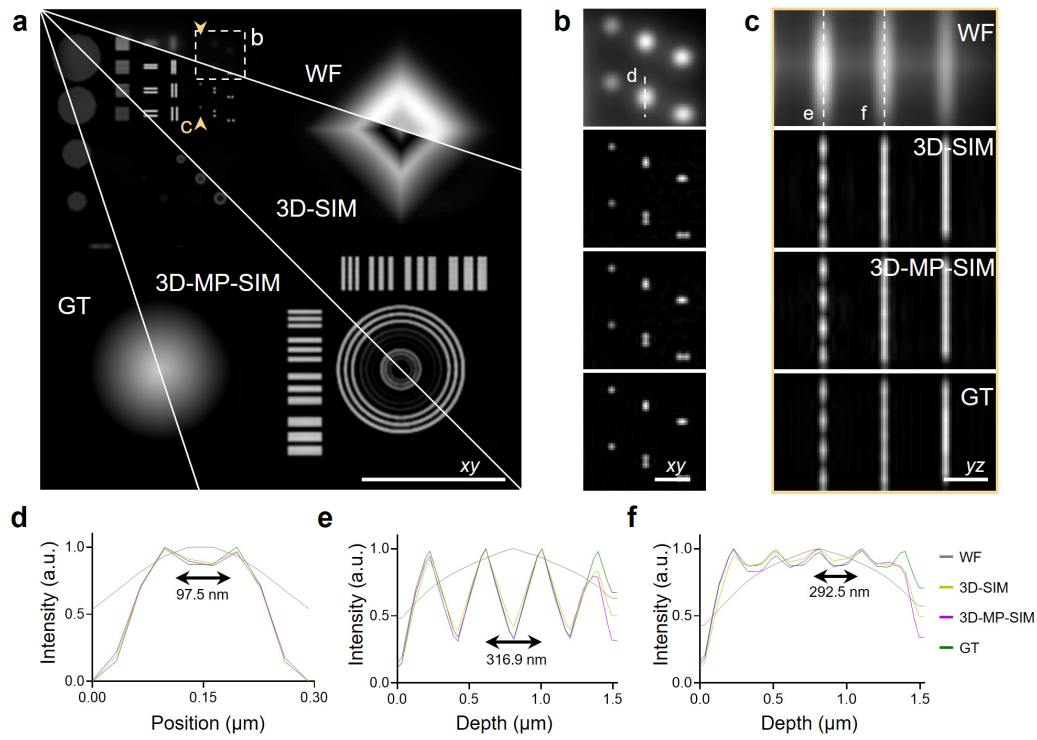
a, Conventional 2D SIM illumination resulting from the interference produced by the ± 1 diffraction orders, where the lateral phase remains the same at the focal plane before and after pattern movement. **b**, 2D SIM illumination resulting from the interference produced by the 0 and +1 diffraction orders, where the axial phase shift introduced by the optical delay line is converted into an easily measurable lateral phase shift.



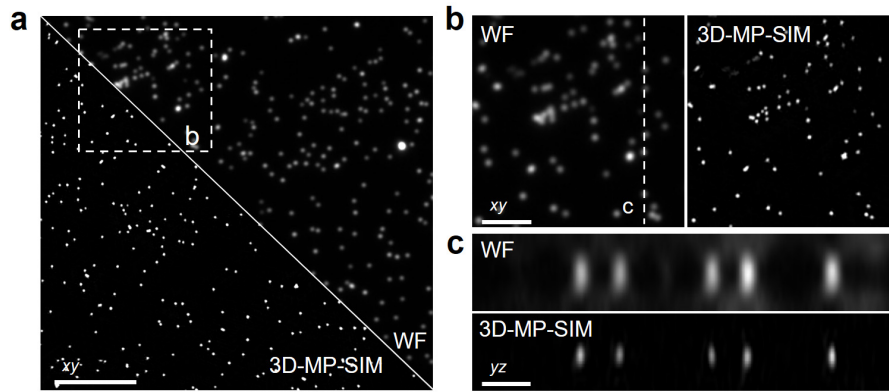
Supplementary Fig. 4: Time sequence of system synchronization. The SLM functions as the master, with its output triggering the sCMOS camera. Then, the sCMOS camera triggered the AOTF to activate the laser. The HS-LCVR changes voltage upon detecting the falling edge of the SLM five times. Once the falling edge count reaches 15, the piezo stage for axial phase shift is adjusted by a predefined $s/2$.



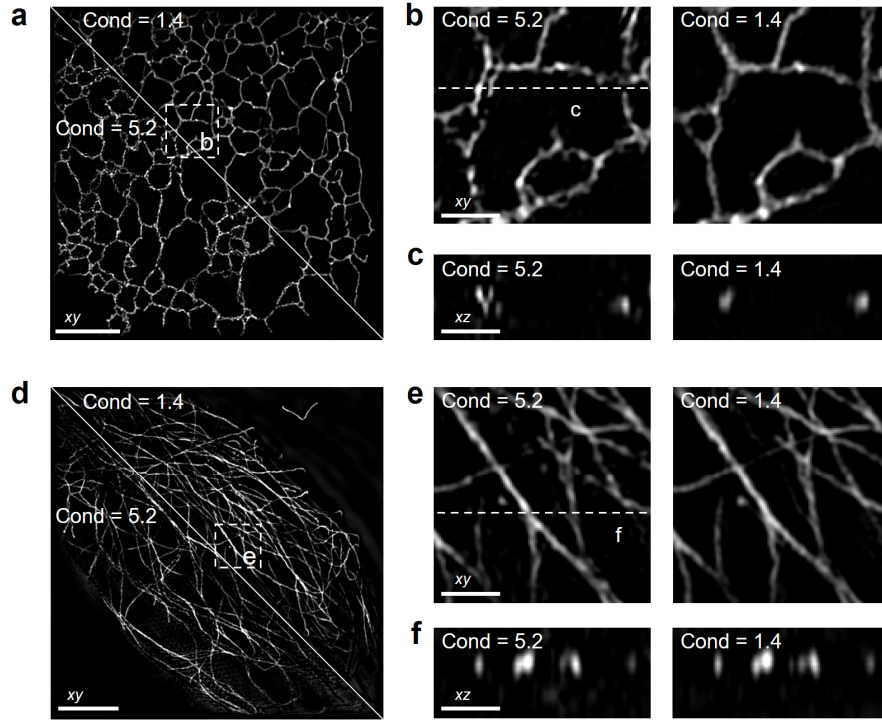
Supplementary Fig. 5: Registration of eight-planes raw data. **a**, Moving stage to obtain registration beads data from areas 1-8 in the camera, where different colors represent eight camera regions having different distributions. **b**, Average intensity projection for each region, with the 5th region as the reference area, to calculate registration matrix. **c**, Eight planes of raw data collected by 3D-MP-SIM. The size of the circles represents those eight camera regions captured different focal planes, and the rightmost overlap image reflects the lateral misalignment across the different regions. **d**, Applying the registration matrix to 3D-MP-SIM raw data to generate aligned data for reconstruction.



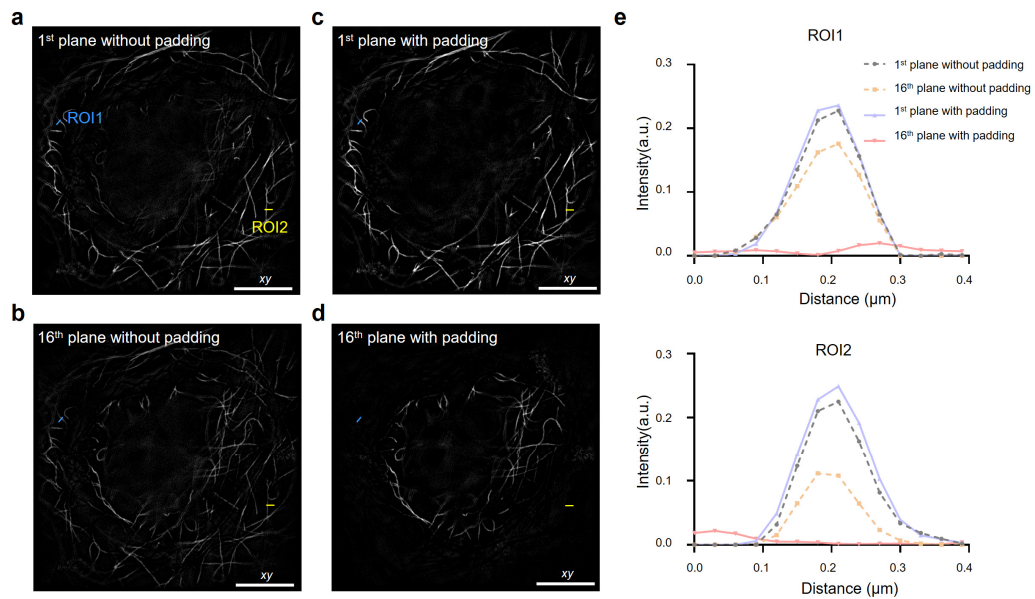
Supplementary Fig. 6: Simulation data reconstructed with a seven-dimension matrix in 3D-MP-SIM. **a**, A lateral distribution of the simulation data, illustrating the lateral performances of the WF, 3D-SIM, 3D-MP-SIM, and the ground truth (GT). **b**, Magnified x-y cross-sections of the boxed regions in **a**, from top to bottom are WF, 3D-SIM, 3D-MP-SIM, and the ground truth. **c**, Magnified x-z cross-sections between arrowheads in **a**. **d**, Lateral intensity profiles of the two adjacent dots highlighted by the dashed line in **b**, demonstrating that the lateral resolvability of 3D-MP-SIM is comparable to that of 3D-SIM. **e**, **f**, Axial intensity profiles of multiple adjacent dots highlighted by the dashed lines in **c**, demonstrating that the axial resolvability of 3D-MP-SIM is comparable to that of 3D-SIM. Scale bars: 5 μm (**a**); 500 nm (**b**, **c**). a.u., arbitrary units. All cross-section slices are 32.5 nm thick.



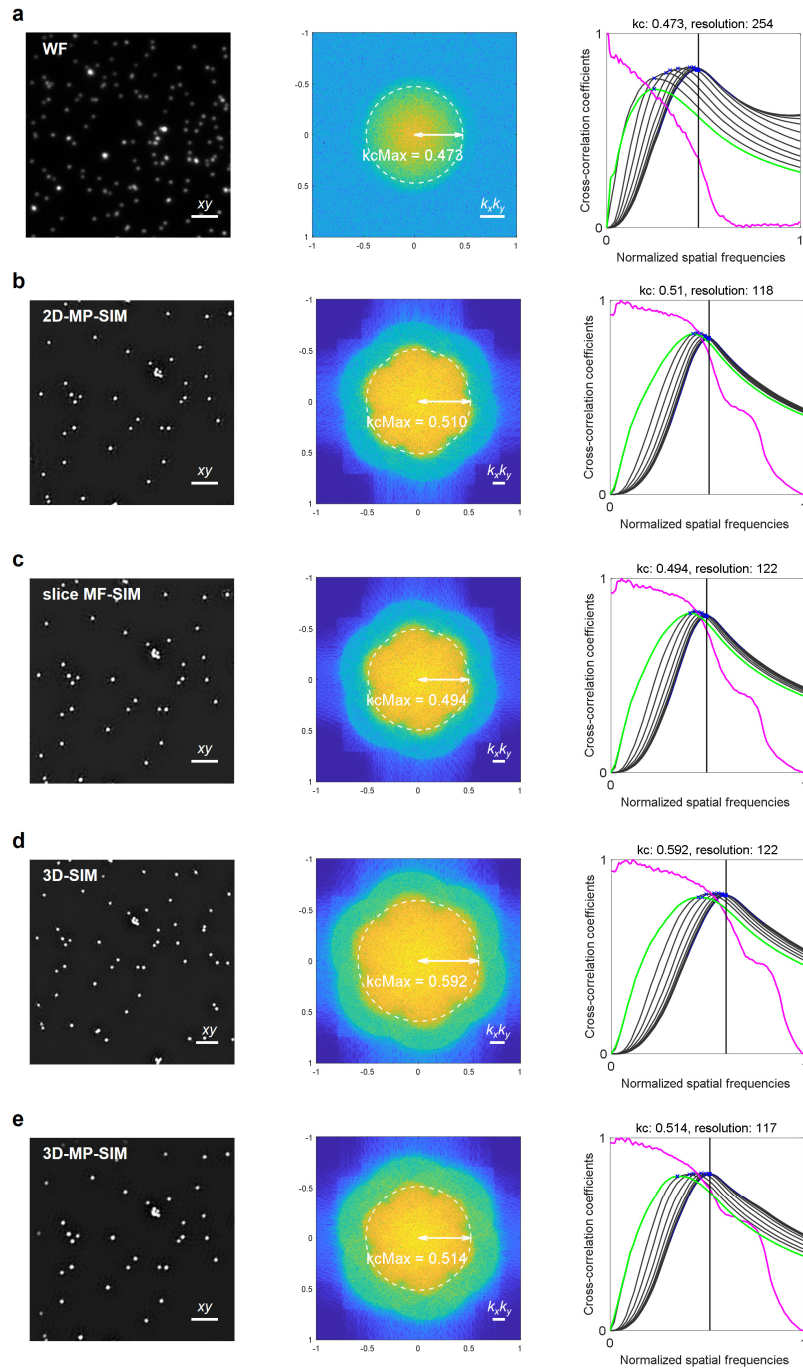
Supplementary Fig. 7: Fluorescent beads reconstructed with a seven-dimensional matrix in 3D-MP-SIM. **a**, Lateral image of a field of 100-nm fluorescent beads, divided into two subfields, showing the differences in the lateral resolution between WF (top-right) and 3D-MP-SIM (bottom-left). **b**, Magnified display of boxed region in **a** for both WF and 3D-MP-SIM. **c**, y-z cross-sections along the dashed line in **b** for WF and 3D-MP-SIM. Scale bars: 5 μm (**a**); 2 μm (**b**); 1 μm (**c**). All cross-section slices are 30 nm thick.



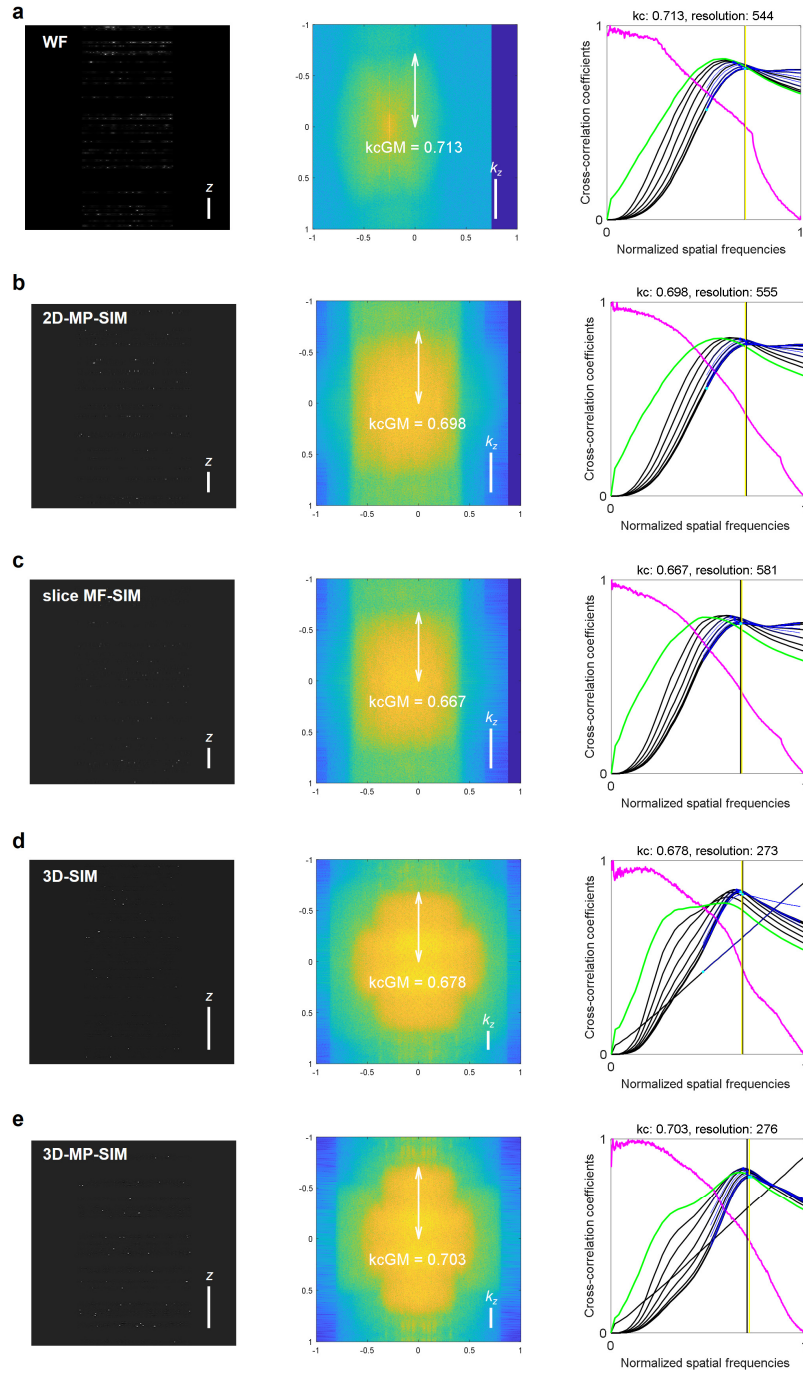
Supplementary Fig. 8: Comparison of the biological samples reconstructed with a seven-dimensional separation matrix or a two-step separation process in 3D-MP-SIM. **a**, Lateral image of the ER structures in a live COS-7 cell, showing the difference between the reconstruction by the two-step separation (top-right, Cond=1.4) and reconstruction by $\mathbf{M}_{7 \times 7}$ matrix separation (bottom-left, Cond=5.2). **b**, Magnified display of the boxed region in **a**. **c**, The x-z cross-sections along the dashed lines in **b**. **d**, Lateral image of immunolabelled microtubules in a fixed U2OS cell, showing the difference between the reconstruction by the two-step separation (top-right, Cond=1.4) and the reconstruction by $\mathbf{M}_{7 \times 7}$ matrix separation (bottom-left, Cond=5.2). **e**, Magnified display of the boxed region in **d**. **f**, The x-z cross-sections along the dashed line in **e**. Scale bars: 5 μm (**a**, **d**); 1 μm (**b**, **c**, **e**, **f**). All cross-section slices are 30 nm thick.



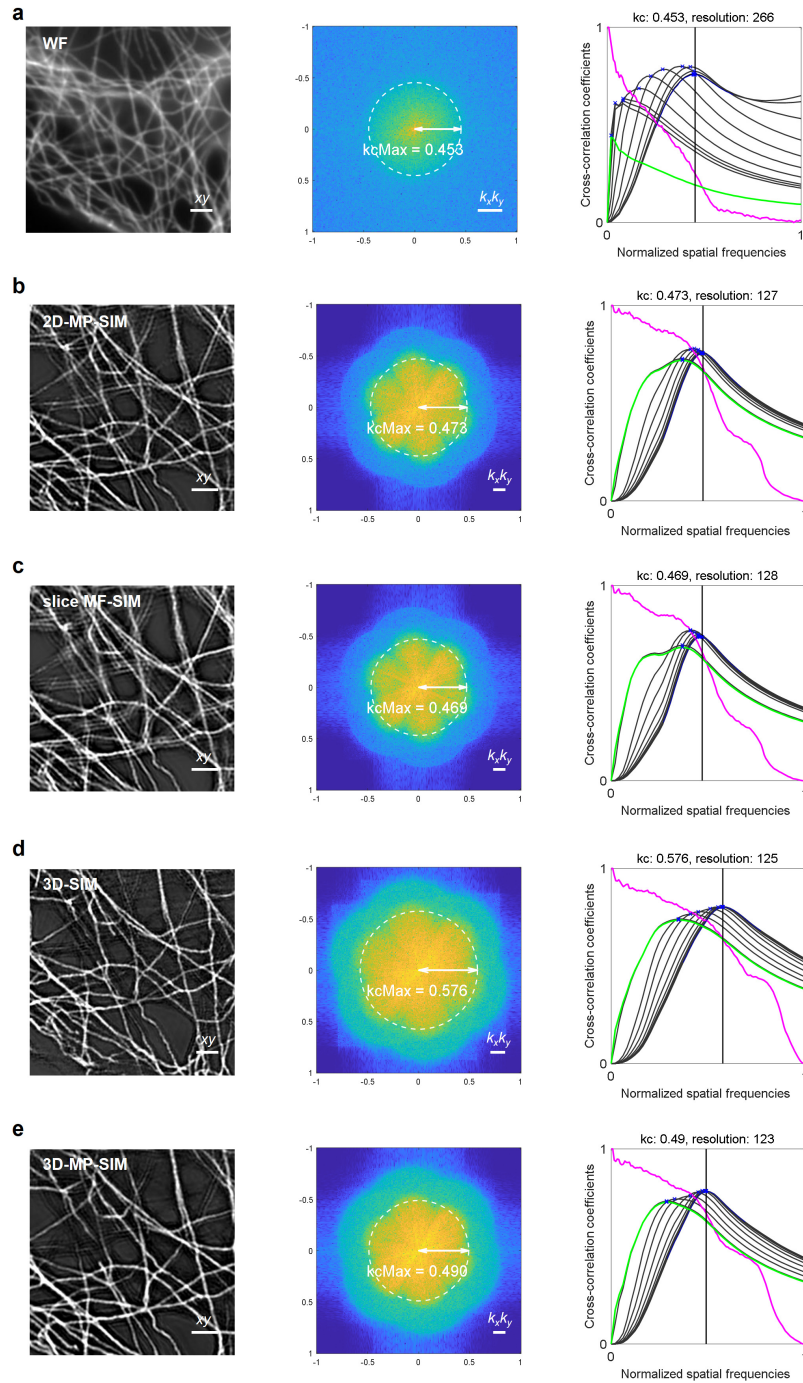
Supplementary Fig. 9: Roll-over artifacts and the effectiveness of reconstruction using padding in microtubules. **a, b**, 1st and 16th planes of microtubules (immunolabelled with anti- α -tubulin and anti- β -tubulin antibodies) reconstructed without padding. **c, d**, 1st and 16th planes reconstructed with padding. **e**, Intensity along the blue and yellow lines demonstrates the contrast between reconstructions with and without padding. Scale bars: 5 μm (**a-d**). a.u., arbitrary units. All cross-section slices are 30 nm thick.



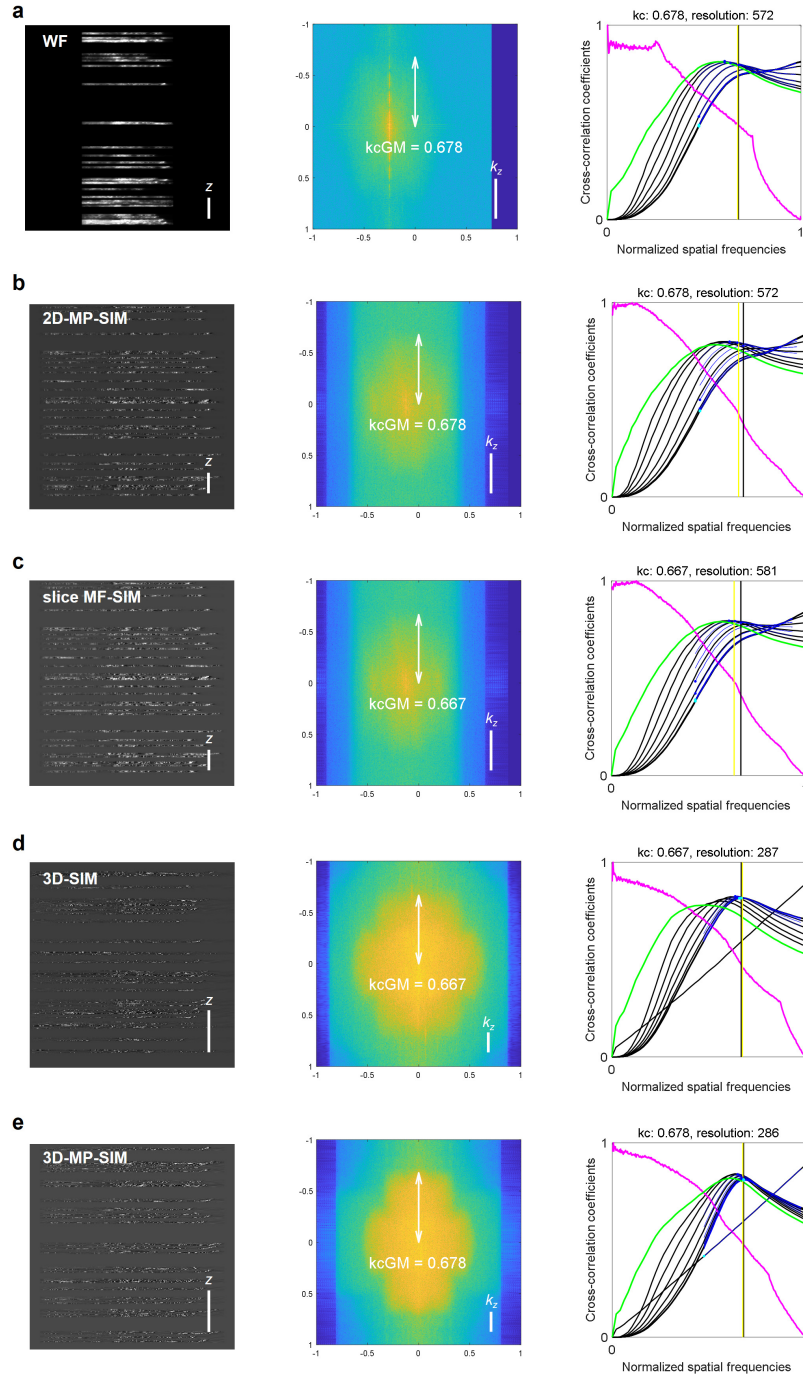
Supplementary Fig. 10: Lateral resolution estimation of each imaging mode on 100-nm fluorescent beads utilizing decorrelation analysis. a-e, Representative image slices of the 100-nm fluorescent beads imaged with WF and four SIM modes (left columns). Decorrelation analysis results (right columns) are displayed for each imaging mode, with corresponding spectra plotted on a logarithmic scale (middle columns). Here, kcMax denotes the maximum spatial frequency. The lateral resolution is determined by the formula ($2 \times \text{pixelsize}/\text{kcMax}$), with the estimated resolution marked in the top of the decorrelation functions. Scale bars: $2 \mu\text{m}$ (left columns); $1/500 \text{ nm}^{-1}$ (middle columns).



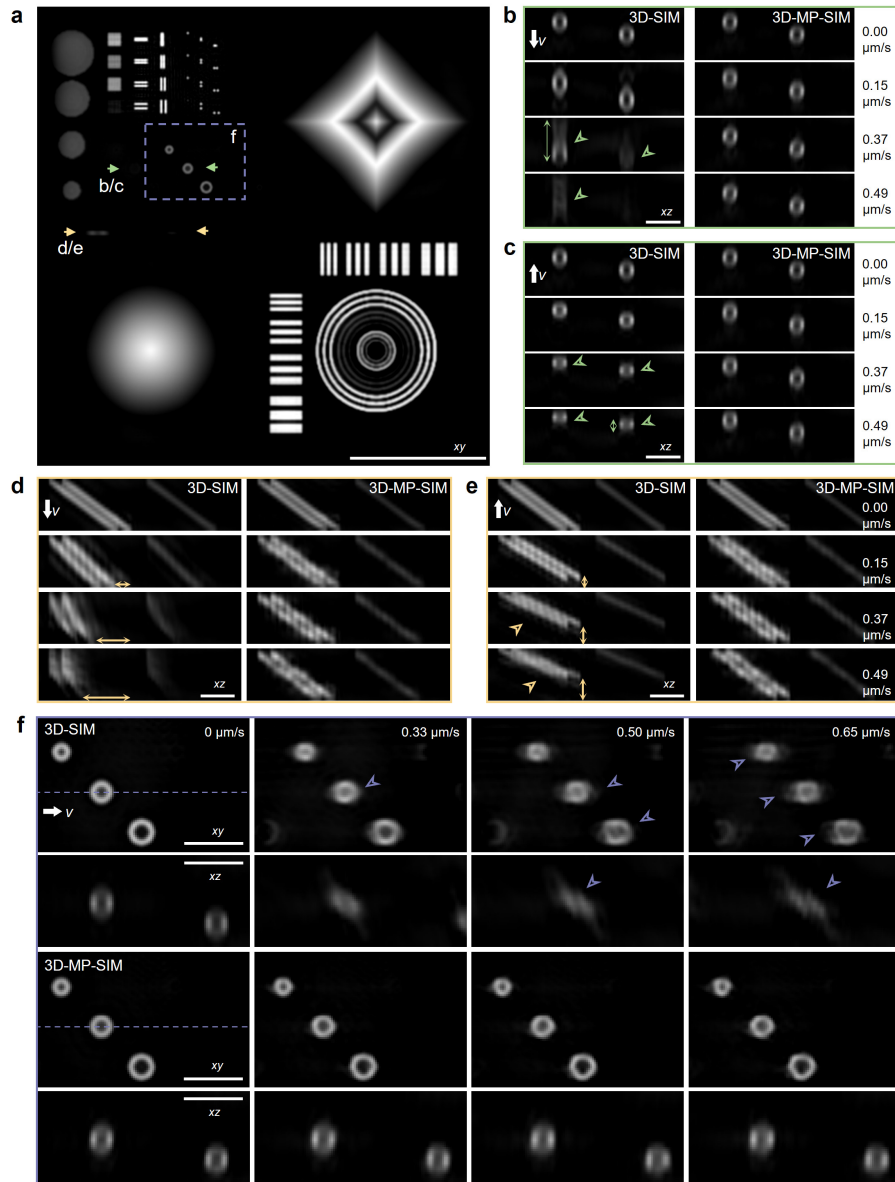
Supplementary Fig. 11: Axial resolution estimation of each imaging mode on 100-nm fluorescent beads utilizing decorrelation analysis. **a-e**, Representative concatenated y-z cross-sections slice of the 100-nm fluorescent beads imaged with WF and four SIM modes (left columns). Decorrelation analysis results (right columns) are displayed for each imaging mode, with corresponding spectra plotted on a logarithmic scale (middle columns). Here, kcGM denotes the maximum spatial frequency. The axial resolution is determined by the formula ($2 \times \text{pixelsize}/\text{kcGM}$), with the estimated resolution marked in the top of the decorrelation functions. Scale bars: 20 μm (left columns); 1 μm^{-1} (middle columns).



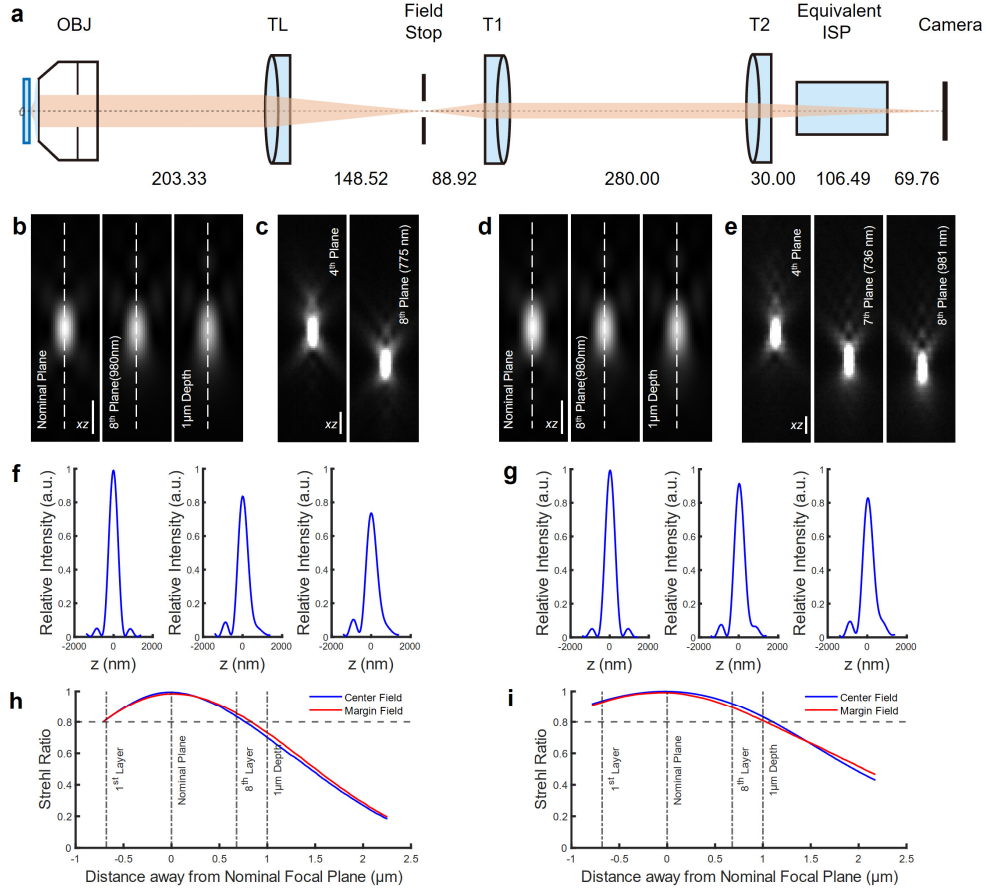
Supplementary Fig. 12: Lateral resolution estimation of each imaging mode on microtubules utilizing decorrelation analysis. **a-e**, Representative image slices of the microtubules imaged with WF and four SIM modes (left columns). Decorrelation analysis results (right columns) are displayed for each imaging mode, with corresponding spectra plotted on a logarithmic scale (middle columns). Here, kc_{Max} denotes the maximum spatial frequency. The lateral resolution is determined by the formula ($2 \times \text{pixelsize}/kc_{Max}$), with the estimated resolution marked in the top of the decorrelation functions. Scale bars: $2 \mu\text{m}$ (left columns); $1/500 \text{ nm}^{-1}$ (middle columns).



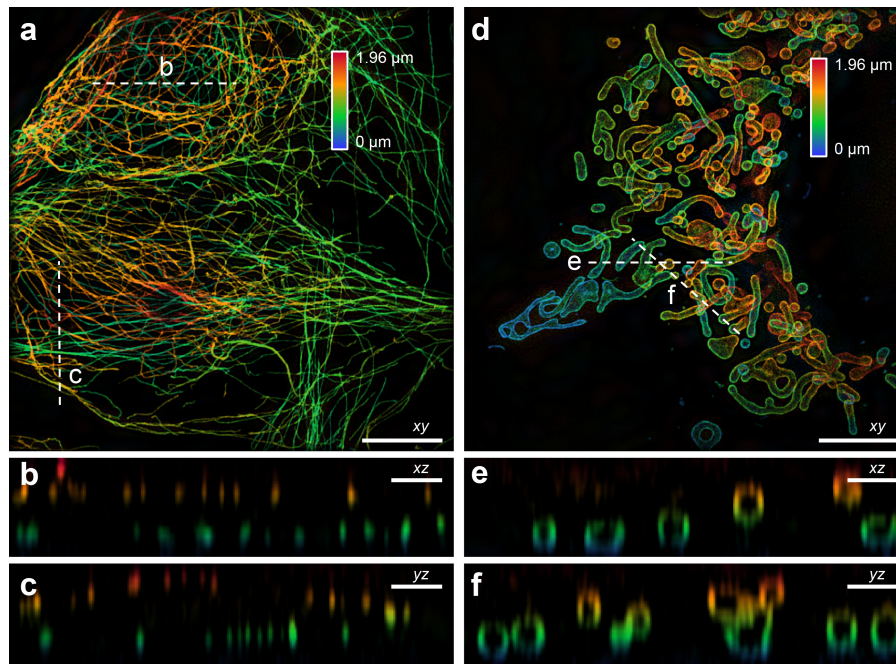
Supplementary Fig. 13: Axial resolution estimation of each imaging mode on microtubules utilizing decorrelation analysis. **a-e**, Representative concatenated y-z cross-sections slice of the microtubules imaged with WF and four SIM modes (left columns). Decorrelation analysis results (right columns) are displayed for each imaging mode, with corresponding spectra plotted on a logarithmic scale (middle columns). Here, kcGM denotes the maximum spatial frequency. The axial resolution is determined by the formula ($2 \times \text{pixelsize}/\text{kcGM}$), with the estimated resolution marked in the top of the decorrelation functions. Scale bars: 20 μm (left columns); 1 μm^{-1} (middle columns).



Supplementary Fig. 14: Comparative simulation of motion artifacts in 3D-SIM and 3D-MP-SIM. **a**, The 3D-MP-SIM reconstruction of the simulated data with a 5 ms exposure time, a 5.39 ms interval between exposures, and a 0 $\mu\text{m/s}$ movement. **b** and **c**, Axial comparison of the ellipsoid shells between green arrowheads in **a**, with different axial speeds indicated on the right. For 3D-SIM imaging with 10 ms movement between planes, the ellipsoid shells appear elongated or contracted when moving along or opposite to the sample stage movement, as indicated with green arrowheads. In contrast, 3D-MP-SIM maintains the structure even at the fastest moving speed of 0.49 $\mu\text{m/s}$. **d**, **e**, Axial comparison of tilted lines between yellow arrowheads in **a**, with different axial speeds indicated in the right panel. The tilt angle changes with the same moving speed in 3D-SIM, while it shows minimal changes in 3D-MP-SIM. **f**, Comparison of the images of the ellipsoid shells at different lateral speeds. The x-z cross-sections of the ellipsoid shells demonstrate the motion artifacts in 3D-SIM, while 3D-MP-SIM shows minimal artifacts. Scale bars: 5 μm (**a**); 1 μm (**b-f**). All cross-section slices are 32.5 nm thick.



Supplementary Fig. 15: Reduction of Strehl ratio as the focal plane deviates from the nominal focal plane. **a**, Layout of the detection system containing a 60×/1.49 NA oil immersion objective, the tube lens ($f_{TL} = 200$ mm), the field stop, the telescope lenses T1 ($f_1 = 100$ mm) and T2 ($f_2 = 180$ mm) and the glass block of specific thickness (the equivalent replacement of the ISP) with the camera. The distances between each element are indicated in millimeters. Simulations were conducted in Zemax OpticStudio 2023 R1.00. **b**, The simulated PSFs of the x-z cross-sections corresponding to focal planes at different depths from the nominal focal plane under the effective detection NA of 1.333. The nominal focal plane is optimized at the middle plane of 4th and 5th planes. **c**, The experimental PSFs of the x-z cross-sections corresponding to the 4th plane and 8th plane under oil immersion objective with 193.8 nm interplane distance. **d**, The simulated PSFs of the x-z cross-sections at different depths under the maximum detection NA of 1.27. The nominal focal plane is optimized at the middle plane of 4th and 5th planes. **e**, The experimental PSFs of the x-z cross-sections corresponding to the 4th plane, 7th plane and 8th plane under water immersion objective with 245.3 nm interplane distance. **f**, Axial intensity profiles highlighted by the dashed lines in **b**. **g**, Axial intensity profiles highlighted by the dashed lines in **d**. **h**, Reduction of Strehl ratio as the focal plane deviates from the nominal focal plane under detection NA of 1.333. The Strehl ratio remains at a relatively high level (>0.8) within 8th plane. **i**, Reduction of Strehl ratio as the focal plane deviates from the nominal focal plane under detection NA of 1.27. The Strehl ratio remains at a relatively high level (>0.8) within 1 μm away from the nominal focal plane. Scale bars: 500 nm (**b-e**). a.u., arbitrary units. Margin field: 13.3 x 13.3 μm from the center field at object plane.



Supplementary Fig. 16: Imaging microtubules and mitochondria using 3D-MP-SIM with 245.3 nm z-spacing. **a**, MIP of immunolabelled microtubules in a fixed COS-7 cell imaged with 3D-MP-SIM; **b**, **c**, Magnified x-z cross-sections (**b**) and y-z cross-sections (**c**) along the dashed lines marked in **a**. **d**, MIP of the mitochondria transfected with mStayGold-OMP25 and TOM20-mStayGold in a fixed COS-7 cell imaged with 3D-MP-SIM. **e**, **f**, Magnified x-z cross-sections (**e**) and y-z cross-sections (**f**) along the dashed lines marked in **d**. Scale bars: 5 μm (**a**, **d**); 1 μm (**b**, **c**, **e**, **f**). All cross-section slices are 30 nm thick.

Supplementary Table 1: Resolution estimation from beads and microtubules using decorrelation analysis. n: number of x-y cross-sections or concatenated y-z cross-sectional images used for each statistic; s.d.: standard deviation. The data were from four (beads) and three (microtubules) independent experiments.

Figure #	n	Decorrelation resolution (nm)	Wide-field		2D-MP-SIM		slice MF-SIM		3D-SIM		3D-MP-SIM	
			Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Fig.2e	64	Lateral	248.63	9.82	116.46	2.42	118.47	3.02	119.62	2.09	112.94	3.40
	48	Axial	543.01	10.55	562.61	17.32	576.16	8.50	274.06	4.49	273.58	4.84
Fig.2i	48	Lateral	266.42	2.44	126.22	1.95	129.14	1.84	125.09	5.33	122.08	2.86
	36	Axial	556.65	15.65	572.48	13.62	578.68	18.51	288.21	6.59	283.68	2.38

Supplementary Table 2: Resolution estimation from beads and microtubules using FWHM. n: number of beads or sub-regions in the microtubule images used for each statistic; s.d.: standard deviation. The data were from four (beads) and three (microtubules) independent experiments.

Figure #	n	FWHM(nm)	Wide-field		2D-MP-SIM		slice MF-SIM		3D-SIM		3D-MP-SIM	
			Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Extended Data Fig.2a	150	Lateral	244.38	10.86	108.14	4.36	114.09	5.74	117.83	3.53	113.92	4.94
	150	Axial	499.81	11.88	476.13	12.22	497.88	10.65	303.32	8.65	292.26	8.81
Extended Data Fig.2b	50	Lateral	247.37	9.16	101.23	9.16	103.63	9.83	110.36	7.02	108.51	8.21
	50	Axial	565.45	39.90	484.57	30.10	508.69	37.42	297.01	15.64	282.18	10.33

Supplementary Table 3: Data acquisition and reconstruction parameters. $W \times H \times D \times C$ represents voxel size in x, y, z dimensions and the number of color channels.

Figure	Imaging mode	Sample / Structure	Laser Intensity (W/cm ²)	Volume size W x H x D x C (Z step size)	Time points	Exposure time(ms)	Volume time(s)	Total Acquisition time(s)	Wiener parameter	Z-padding planes
Fig.2 a, b	2D-MP-SIM	100-nm yellow-green beads (488 nm)	61	292x292x8x1 (193.8 nm)	1	10	0.18	0.18	1×10^{-3}	None
	slice MP-SIM	100-nm yellow-green beads (488 nm)	53	292x292x8x1 (193.8 nm)	1	10	0.31	0.31	1×10^{-3}	None
	3D-SIM	100-nm yellow-green beads (488 nm)	7	292x292x16x1 (92.5 nm)	1	10	5.10	5.10	1×10^{-5}	8
	3D-MP-SIM	100-nm yellow-green beads (488 nm)	53	292x292x8x1 (193.8 nm)	1	10	0.61	0.61	1×10^{-5}	4
Fig.2 f, g, h	2D-MP-SIM	Fixed U2OS cells / microtubules	61	462x462x8x1 (193.8 nm)	1	10	0.18	0.18	1×10^{-2}	None
	slice MP-SIM	Fixed U2OS cells / microtubules	53	462x462x8x1 (193.8 nm)	1	10	0.31	0.31	1×10^{-2}	None
	3D-SIM	Fixed U2OS cells / microtubules	7	462x462x16x1 (95.8 nm)	1	10	5.10	5.10	1×10^{-4}	8
	3D-MP-SIM	Fixed U2OS cells / microtubules	53	462x462x8x1 (193.8 nm)	1	10	0.61	0.61	1×10^{-4}	4
Fig.2 j, k, l, m	2D-MP-SIM	Fixed COS-7 cells/ Outer mitochondrial membranes	61	462x462x8x1 (193.8 nm)	1	10	0.18	0.18	1×10^{-2}	None
	slice MP-SIM	Fixed COS-7 cells/ Outer mitochondrial membranes	53	462x462x8x1 (193.8 nm)	1	10	0.31	0.31	1×10^{-2}	None
	3D-SIM	Fixed COS-7 cells/ Outer mitochondrial membranes	7	462x462x16x1 (92.5 nm)	1	10	5.10	5.10	1×10^{-4}	8
	3D-MP-SIM	Fixed COS-7 cells/ Outer mitochondrial membranes	53	462x462x8x1 (193.8 nm)	1	10	0.61	0.61	1×10^{-4}	4
Fig.3 a, b, c	3D-SIM	Living COS-7 cells/ Late endosomes	7	462x462x16x1 (92.5 nm)	50	5	2.69	134.50	1×10^{-4}	8
	3D-MP-SIM	Living COS-7 cells/ Late endosomes	53	462x462x8x1 (193.8 nm)	50	5	0.31	15.50	1×10^{-4}	4
Fig.3 d, e, f	3D-MP-SIM	Living COS-7 cells/ Outer mitochondrial membranes	53	462x462x8x1 (193.8 nm)	67	5	0.31	20.77	1×10^{-4}	4
Fig.3 g, h, i	3D-MP-SIM	Living COS-7 cells/ Endoplasmic reticulum	53	462x223x8x1 (193.8 nm)	142	1	0.09	12.92	1×10^{-4}	4
Fig.4 a, b, c	3D-MP-SIM	Living COS-7 cells/ The inner and outer mitochondrial membranes	488: 53	462x462x8x2 (193.8 nm)	72	5	0.69	49.68	1×10^{-4}	4
			560: 85 *							
Fig.4 d, e, f, g	3D-MP-SIM	Living COS-7 cells/ Outer mitochondrial membranes and endoplasmic reticulum	488: 53	462x462x8x2 (193.8 nm)	56	5	0.69	38.64	1×10^{-4}	4
			560: 102 **							

*(increased by 0.1% each SIM image until 170 W/cm²)

** (increased by 0.1% each SIM image until 204 W/cm²)