

Implementation of a 4Pi-SMS super-resolution microscope

Jingyu Wang^{1,8}, Edward S. Allgeyer^{2,8}, George Sirinakis^{1,2}, Yongdeng Zhang^{1,3}, Kevin Hu^{1,3,4}, Mark D. Lessard^{1,3}, Yiming Li^{5,6}, Robin Diekmann^{1,5}, Michael A. Phillips^{1,7}, Ian M. Dobbie^{1,7,✉}, Jonas Ries^{1,5,✉}, Martin J. Booth^{1,✉} and Joerg Bewersdorf^{1,3,4,✉}

The development of single-molecule switching (SMS) fluorescence microscopy (also called single-molecule localization microscopy) over the last decade has enabled researchers to image cell biological structures at unprecedented resolution. Using two opposing objectives in a so-called 4Pi geometry doubles the available numerical aperture, and coupling this with interferometric detection has demonstrated 3D resolution down to 10 nm over entire cellular volumes. The aim of this protocol is to enable interested researchers to establish 4Pi-SMS super-resolution microscopy in their laboratories. We describe in detail how to assemble the optomechanical components of a 4Pi-SMS instrument, align its optical beampath and test its performance. The protocol further provides instructions on how to prepare test samples of fluorescent beads, operate this instrument to acquire images of whole cells and analyze the raw image data to reconstruct super-resolution 3D data sets. Furthermore, we provide a troubleshooting guide and present examples of anticipated results. An experienced optical instrument builder will require ~12 months from the start of ordering hardware components to acquiring high-quality biological images.

Introduction

4Pi-single-molecule switching (SMS) microscopy is a super-resolution fluorescence imaging technique that combines two concepts: (i) SMS fluorescence microscopy, also known as single-molecule localization microscopy (SMLM)—e.g., (fluorescence) photoactivation localization microscopy ((F) PALM) and (direct) stochastic optical reconstruction microscopy ((d)STORM)—in which individual blinking fluorescent molecules are sparsely activated and localized with sub-diffraction accuracy, and (ii) 4Pi detection, in which fluorescence collected by two opposing objectives, sandwiching a sample, is interferometrically combined. This concept has been realized in a number of similar systems over the last decade^{1–4}. Compared with single-objective SMLM, 4Pi detection provides a $\sqrt{2}$ improved localization precision in the XY direction (focal plane) due to twice the photon-collection efficiency. More importantly, it yields an approximately sixfold resolution improvement in the Z (axial) direction because of the axial interference pattern, which features $\lambda/4$ -wide interference maxima. Resolution values of ~20 nm have been demonstrated in the lateral dimensions and down to 10 nm along the optical axis. This has been achieved over 3D volumes ranging in thickness from several hundred nanometers up to 10 microns, when using adaptive optics⁴. In this protocol, we provide detailed instructions on how to assemble, test and operate a 4Pi-SMS microscope.

4Pi detection, combined with SMLM, was first implemented with three interference channels as interferometric PALM (iPALM)¹ and, later, with four interference channels as 4Pi-SMS². The foundation of this protocol article is an instrument first reported in 2016⁴, which has been used in a range of applications since then, revealing nanoscale features in whole mammalian cells and bacteria^{4–9} and in material sciences¹⁰. The instrument described here has been improved over the original instrument with respect to its mechanical stability, piezo actuators and new operating and analysis software. These improvements are the result of a joint development effort by research laboratories at the University of Cambridge, the European Molecular Biology Laboratory (EMBL)

¹Department of Engineering Science, University of Oxford, Oxford, UK. ²The Gurdon Institute, University of Cambridge, Cambridge, UK. ³Department of Cell Biology, Yale School of Medicine, New Haven, CT, USA. ⁴Department of Biomedical Engineering, Yale University, New Haven, CT, USA. ⁵European Molecular Biology Laboratory, Heidelberg, Germany. ⁶Department of Biomedical Engineering, Southern University of Science and Technology, Shenzhen, China. ⁷Micron Advanced Bioimaging Unit, Department of Biochemistry, University of Oxford, Oxford, UK. ⁸These authors contributed equally: Jingyu Wang, Edward S. Allgeyer. ✉e-mail: ian.dobbie@bioch.ox.ac.uk; jonas.ries@embl.de; martin.booth@eng.ox.ac.uk; joerg.bewersdorf@yale.edu

Heidelberg, the University of Oxford and Yale University. Our protocol summarizes the combined experience of instrument setup, alignment, calibration and operation at these four institutions.

Although this protocol and the associated resources contain the information necessary to construct and use a 4Pi-SMS microscope, we would like to point out that it is a complex system requiring significant effort and expertise in both the construction of complex optical instruments and application of super-resolution microscopy. We estimate that a full-time, experienced optical instrument builder would require ~12 months to implement the instrument described here. All part lists, blueprints, models and software can be found on GitHub: <https://github.com/4Pi-SMS-consortium>. In addition, there are ongoing development efforts across multiple institutions, and we encourage readers to reach out to check for any updates or to ask questions before or during the build process.

Conceptual description of the 4Pi-SMS setup

In brief, 4Pi-SMS microscopy is an interference-based wide-field imaging method. Fluorescence emitted by a single molecule (fluorophore) is collected by two opposing objectives with a common focus and follows two identical, but separate, beam paths until it is combined with a beam splitter cube and interferes. After combination, the fluorescence emission is relayed onto a camera, with appropriate optics, where it forms four images representing different interference phases. These images are recorded and later analyzed to reconstruct a 3D super-resolution image.

In this approach, a molecule's lateral XY position is determined using a conventional SMLM technique by fitting a model function to a single-molecule image. The 4Pi interference effect is used to find each molecule's axial Z position with high precision. To achieve the required interference contrast, the optical path length difference between the two optical paths must be within the coherence length of a single emitter, typically 5–10 μm . Furthermore, because the 4Pi interference pattern repeats every $\lambda/2$ (~250 nm), relying on interference intensity alone may result in Z-position misassignment. To address this limitation, astigmatism is deliberately added to each light path after each objective^{4,11}. The point spread function (PSF) distortion introduced by astigmatism allows the molecule's Z-position to be assigned to one peak of the 4Pi interference pattern and thereby eliminates ambiguity in axial localization. Adaptive optics is used to shape the PSF through the introduction of astigmatism and to correct for specimen-induced aberrations that vary with depth.

Optical overview

The microscope presented here is centered around two opposing 100 \times /1.35 numerical aperture (NA) silicone oil immersion objective lenses (Olympus, UPLSAPO 100XS), OBJ0 and OBJ1 in Fig. 1. Critically, these objectives must have closely matched magnification (within 0.3%) to allow for alignment of the two imaged fields of view for interference. Each objective lens is immediately followed by a quarter-wave plate (QWP) (Fig. 1a, QWP0-1) set at 45° with respect to the plane of the setup^{2,4}. The quarter-wave plates ensure that the emitted light is split in both beam paths into s- and p-polarization components of equal intensity, independent of the initial polarization orientation.

Each objective's back pupil plane is imaged onto a deformable mirror (DM) (Fig. 1a, lenses L1 and L3 for OBJ0 and lenses L2 and L4 for OBJ1). The DMs serve three functions: (i) introduce astigmatism to assist in emitter z-position determination, (ii) correct for system aberrations and (iii) compensate for specimen-induced aberrations, particularly depth-dependent spherical aberrations.

Each DM is followed by an optical wedge pair and flat (Fig. 1a, W0 and W1)^{2,4}. In one light path, a pair of custom quartz wedges with the axes oriented to match the polarization channels, with one wedge mounted on a translation stage, acts as a variable retardance waveplate. The stationary wedge is bonded to a BK7 flat. In the other light path, a similar arrangement is introduced with the roles of quartz and BK7 switched. The quartz and BK7 flats act as offsets for the wedge pairs made from the same material in the opposite beam path. This arrangement allows control over the interference phase of the s- and p-polarization channels via the quartz wedge pair, while the BK7 wedge pair allows for the dispersion of the system to be adjusted. When tuned correctly, this results in an interference phase shift close to $\pi/2$ between each image with minimal differences between color channels.

Subsequently, the light paths are combined with a nonpolarizing 50/50 beam splitter cube (Fig. 1a, NPBS (nonpolarizing beam splitter)). The optical components and two light paths from the sample to the NPBS comprise the interference cavity, as presented in Fig. 1a. The s- and p-components from the cavity's upper path interfere with the light of the same polarization from the lower path. Thus, s- and p-polarized light of different interference phases leaves the NPBS at both sides and propagates through lenses L5 and L0, respectively (Fig. 1a). Subsequently, these light paths are relayed by a series

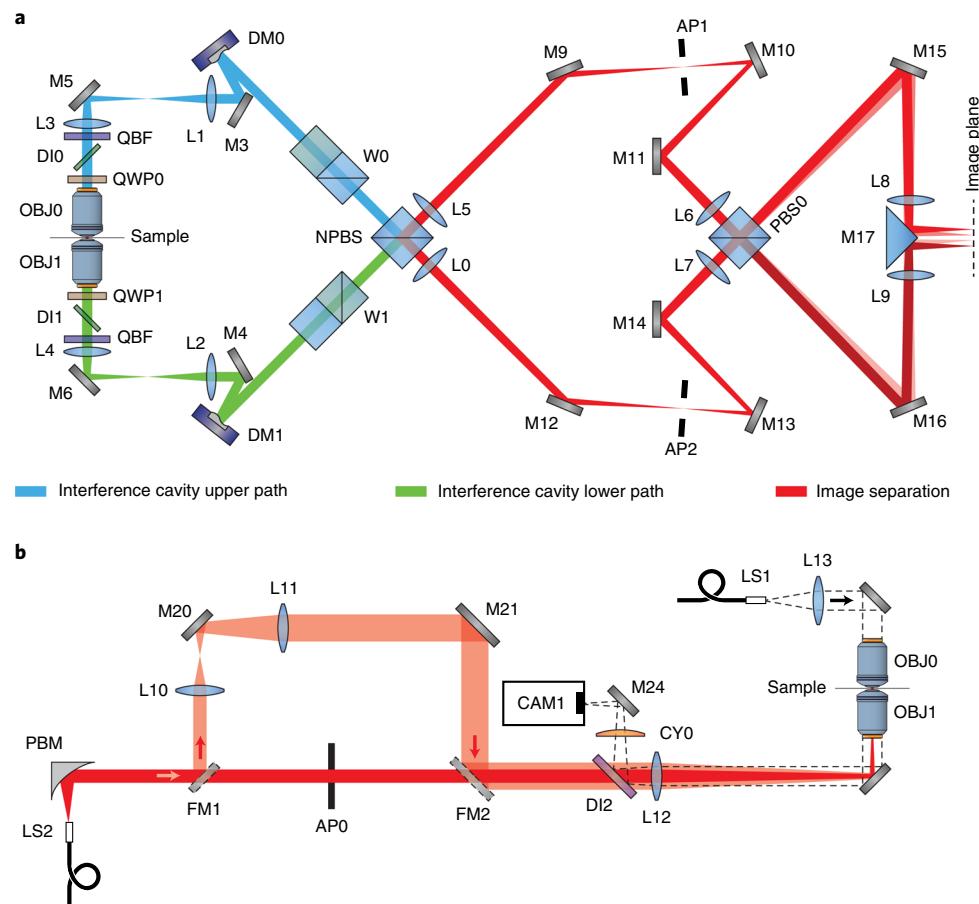


Fig. 1 | 4Pi-SMS detection and excitation path optical overview. **a**, 4Pi-SMS detection light path. Fluorescence emission is collected by two opposing objective lenses (OBJ0 and OBJ1) and then directed into two symmetric, identical upper (blue) and lower (green) beam paths. The respective beam paths traverse broadband achromatic QWPs (QWP0 and QWP1), dichroic mirrors for the introduction of excitation laser light (DIO and DI1), two lens pairs for imaging the objective back pupil plane onto DMs (DM0 and DM1) and finally meet at a nonpolarizing 50/50 beamsplitter cube (NPBS). The light paths from the two objectives to the NPBS comprise the interference cavity. After the NPBS, the beam paths pass several mirrors and lenses before reaching the camera at the end of the image separation path (red). M, mirror; PBS0, polarizing beam splitter cube; QBF, quadband filter; W, wedge. Emphasis Type="Bold">>b, 4Pi-SMS illumination light path (red). Light emitted from a fiber (LS2) is collimated (PBM) and follows a straight path through a rectangular aperture (APO) until it is focused into the back aperture of OBJ1. Alternatively, flip mirrors (FM1 and FM2) direct the beam through a telescope (L10 and L11) to illuminate a large area in the sample (light red). Propagating counter to the illumination path, 940-nm light is launched from a fiber (LS1) and collimated (L13) before passing from OBJ0 to OBJ1 and being separated with a dichroic mirror (DI2), passing a cylindrical lens (CY0) and being focused onto a camera (CAM1) for objective alignment (dashed lines).

of lenses and mirrors to a polarizing beam splitter (PBS) cube (Fig. 1a, PBS0) where the four polarization channels are separated before reaching the camera. This is accomplished by slightly adjusting the location and angle of incidence of the two light paths incident on PBS0 using mirrors M11 and M14 (Fig. 1a). The transmitted and reflected beam paths do not interfere again but are separated such that four images are arranged in a vertical line on the camera sensor (Fig. 1). The optical components and light paths after the NPBS comprise the image separation portion of the detection path as shown in Fig. 1a.

Relative to the emission path, the system's excitation path is mounted on the opposite side of a vertical breadboard, as shown in Fig. 2. An overview of the excitation path's optical layout is presented in Fig. 1b. Excitation light is delivered to the system via a single-mode polarization-maintaining fiber. Light launched from the fiber is collimated and follows a straight path through a rectangular aperture and then a lens that focuses the excitation light into the back aperture of OBJ1 (lower objective). The rectangular aperture is used to limit the illumination area at the sample and thus avoids unnecessary photobleaching of areas not being imaged and reduces background from

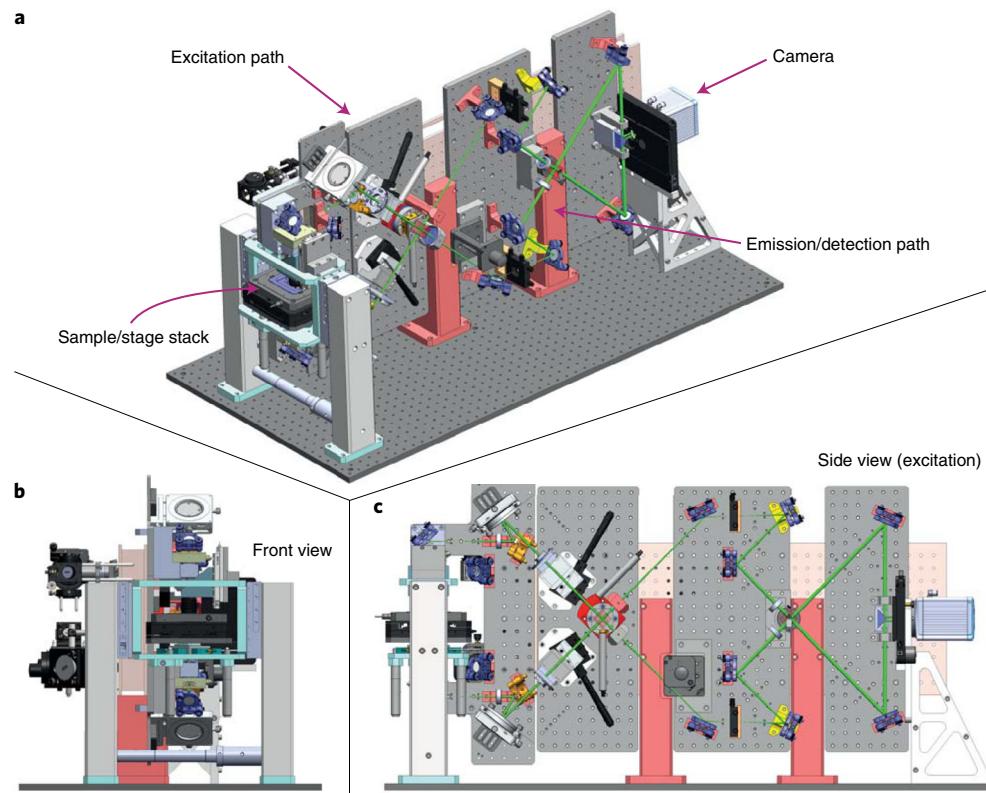


Fig. 2 | 4Pi-SMS system overview. The different sections of the system are indicated in **a**, the system is shown from the front, sample side in **b** and a side view shows the emission/detection path in **c**.

autofluorescence and scattering. The beam size, aperture size and focusing lens have been selected such that the sample is more ‘uniformly’ illuminated (~25% reduction in intensity from the center of the field of view to the edge). The excitation path also includes two flip mirrors that can direct the excitation beam through an alternative path, resulting in a large illumination area at the sample (~100 μm diameter) for sample location and identification.

In addition, the excitation path includes a near-IR laser beam (LS1, Fig. 1b) used for real-time objective alignment. The NIR beam is launched from a fiber and travels in the opposite direction, relative to the excitation light, before being separated by a dichroic mirror and directed onto a camera (CAM1). A cylindrical lens (CY0) in the NIR beam path adds astigmatism before focusing onto the camera. The relative lateral positions of the objectives can be tracked by observing the focused spot on the camera. The astigmatism introduced by the cylindrical lens enables tracking of the axial position of the objectives. Images from this camera are used to correct the objective positions via a closed-loop control.

Mechanical design overview

The mechanical design presented here has three primary targets: (i) maximize vibrational stability, (ii) be reproducible to set up and (iii) be simple to use. In comparison to Huang et al.⁴, the mechanical design has been improved over several iterations to maximize stability and ease of manufacturing, assembly and alignment. Working systems at Yale University, EMBL Heidelberg, University of Cambridge, University of Oxford and ShanghaiTech University, along with previously published images⁴, demonstrate that these goals have been achieved. To ease reproduction, the system is constructed primarily from commercially available optomechanical parts. Custom parts are precision-machined and assembled using guide pins to reduce degrees of freedom and make construction and alignment easier.

The microscope’s mechanical design was originally carried out in Solidworks (Dassault Système) and later moved to Inventor (Autodesk). Any group reproducing this system will need to carefully consult with the existing computer-aided design (CAD) files and part drawings. As such, we refrain from a detailed description of individual components and refer to the part drawings and CAD files for

detail (see Materials for details on how to obtain the microscope CAD files). Reproduction of the physical system is possible from provided design files and part drawings. In the following paragraphs, we provide an overview of the design concept. The primary aim of this protocol is to cover the otherwise intangible details of the microscope: optical alignment, instrument calibration and validation and data collection.

The microscope is based around two core components. First, a tower assembly holds the sample, objective lenses and sample stage stack. Second, a vertical breadboard with mounting holes on both sides holds most of the detection optics and interference cavity beam path on one side and the excitation beam path on the other, as shown in Fig. 2. These two core components are not directly connected, and the tower assembly may be moved for alignment relative to the vertical breadboard section. A vertical geometry was chosen for two reasons. Foremost, using a vertical design allows the sample to remain in a traditional horizontal geometry. If the system were built horizontally the sample would necessarily be vertical, precluding any specimen not tightly adhered to the cover glass (e.g., some tissue sections, some types of cells, egg chambers and embryos). Second, the vertical design allows the excitation and emission paths to be conveniently compartmentalized on a single breadboard. This vertical configuration has been very stable across all systems.

The tower assembly is centered around a stage stack holding the sample and objective lenses. Specifically, the sample is held by a custom sample holder that is mounted to a piezo Z stage (Physik Instrumente (PI), P-541.ZCD) for nanometer axial sample positioning. In turn, the sample Z stage is connected to an XY sample stage (PI, U-751.24). This assembly is supported at three points of contact by three high-resolution linear actuators (PI, M-227.10) that provide millimeters of travel for coarse sample Z-positioning and allows the sample tip/tilt to be adjusted. One objective lens is mounted below the sample (lower objective/OBJ1, Fig. 1) on an XY piezo translation stage (PI, P-612.2SL), while a second objective lens is mounted above the sample (upper objective/OBJ0, Fig. 1) on a long-travel piezo walk stage (PI, N-565.260) that allows nanometer objective axial positioning with millimeters of travel. This arrangement allows the lower objective to be focused by moving the sample in the Z direction. The upper objective can then be brought to the same focal plane. Lateral objective alignment is accomplished by adjusting the XY position of the lower objective. Once both objectives have been brought to a common focus, their positions are fixed, and the sample is moved in XY and Z to the desired field of view. Furthermore, the upper objective may be retracted ~12 mm from the focus position to facilitate easy sample insertion and removal.

These components are all connected in a single unit sandwiched between two vertically oriented linear translation stages (Applied Scientific Imaging, LS-50) held in place by large supporting legs directly connected to the optical table. This allows the entire sample/objective assembly to be translated along the optical axis, and, hence, the path length difference between the two arms of the interference cavity can be tuned for maximum interference.

The second section of the system, centered around the aforementioned vertical breadboard, contains most of the interference cavity and the remainder of the detection optics within the so-called image separation section on the ‘emission side’, as shown in Fig. 2, and the excitation optics on the opposite ‘excitation side’. This breadboard is mounted to the optical table with two large supporting legs. On the emission side, the honeycomb breadboard is connected to four smaller aluminium breadboards via several shoulder screws as position reference fiducials. All individual components are attached to the four small breadboards. This approach allows the removal of subsections of the detection path for correction or adjustment without requiring disassembly of the entire system. The key optical components on the emission side are two DMs for aberration correction, the quartz and BK7 wedge pair and optical flat assemblies for tuning phase and compensating for dispersion, the NPBS where fluorescent emission is recombined and a PBS cube where the four image channels are separated. The camera and an emission filter wheel are directly mounted to the optical table by two supporting legs and are not in direct contact with the vertical breadboard section. The excitation side of the vertical breadboard holds all the optics related to sample excitation and objective alignment.

Although the design presented here requires minimal maintenance once set up, small changes in temperature while imaging may result in path length changes that compromise or eliminate the interference effect, resulting in poor Z resolution. To minimize this effect, a symmetric design is used. The two light paths of the interferometric portion are kept as identical as possible. Thus, any thermal expansion or contraction in one light path is equally experienced by the other, resulting in minimal net change. Inevitably, small changes in path length do occur while imaging, resulting in phase drift over time. This drift is tracked and corrected for in data after processing.

Applications

The presented 4Pi-SMS microscope has demonstrated isotropic sub-20-nm 3D resolution in biological samples $\leq 10\text{ }\mu\text{m}$ thick. It has been successfully used to resolve a wide range of nanostructures in mammalian cells^{4,5,7–9}, including the endoplasmic reticulum, mitochondria, the Golgi apparatus, synaptonemal complexes, etc. New discoveries enabled by 4Pi-SMS microscopy include Nup188 clusters that have been found to form two discrete barrel structures at cilia bases⁵. Furthermore, CatSper1, a calcium channel subunit, was shown to form a two-row structure in mouse sperm cells, which was disrupted in the absence of EFCAB9⁷. In addition, the instrument is not limited to mammalian cells but can be applied to bacteria, in which it has been used, for example, to determine the 3D subcellular distribution of type III secretion nanomachines⁶. Moreover, it has been used in material science to reveal nanoscale crosslink heterogeneities in hydrogels, providing insight into the kinetics of microgel formation¹⁰.

Advantages and limitations

For an overview of the advantages and limitations of super-resolution methods in a biological context, we recommend a recent review by Schermelleh et al.¹². The 4Pi-SMS microscope presented here has several key advantages. Foremost is the superior 3D isotropic resolution. For cell biology applications, 4Pi-SMS provides 3D resolution approaching that of electron microscopy with immunofluorescence labeling specificity. Furthermore, multicolor imaging can easily be added⁹, and any localization-based method using probe switching is compatible with the existing system. This system also allows imaging over the thickness of whole cells with the use of adaptive optics. Acquiring large 3D volumes creates new possibilities for biological applications that require a whole-cell context. When installed in a controlled environment, the system is extremely stable. After initial setup, existing systems require virtually no alignment maintenance and have been found in good working order after 3–4 months of inactivity.

This system does, however, have several limitations. The low temporal resolution of the system precludes the possibility of live cell imaging. That is, this system is compatible only with fixed samples. As a further constraint, using two high-NA opposing objective lenses limits the physical space for a sample and eliminates conventional mounting methods (slides, dishes and welled chambers). Furthermore, the free working distance of the objective lenses also limits sample thickness to less than roughly 100 μm . Thus, only low-confluence cultured cell samples or very thin tissues or tissue sections may be used on this system. For larger cells or thin tissue sections, the limited $20 \times 20\text{ }\mu\text{m}$ field of view may also prove impractical. Data post-processing time is also a limiting factor in imaging throughput and is further complicated by the large data sets typically generated daily. Overall, this system is an excellent choice for applications where superior 3D resolution is required, but for any applications where Z resolution $< 50\text{ nm}$ is not necessary, simpler systems exist and may be a better choice.

Overview of the procedure

Full system setup is a complicated process that will require considerable time and care. Although some portions of the procedure do not need to be carried out in a specific order, many do. In this protocol, we break the process into individual tasks and arrange them linearly. The procedure is built on the assumption that all necessary components are available and ready for use.

Before starting the individual steps in the procedure, all recommendations in ‘Experimental design’ should be carefully reviewed. This section includes guidelines on optical table selection, what alignment laser to use and other information to be considered before setting up the system. ‘Equipment setup’ covers installation of optical components and computer software that must be complete before the procedure is begun.

The procedure begins with the installation of all relevant computer software (Steps 1 and 2) and assembly of all optomechanical components (Steps 3–5). Next, an alignment laser (used as a proxy for fluorescence emission) positioned on the horizontal optical table is coupled into the system via a flip mirror (Steps 6–11). This alignment laser is used to align the most critical section of the microscope, the interference cavity (Steps 12–68). For the best results, it is important to align the excitation path during the cavity alignment portion of the protocol, specifically during objective installation. As such, the procedure follows this progression, and the excitation path is aligned in Steps 69–101.

For alignment of the image separation path (Fig. 1a), the alignment laser is used (Steps 102–141). However, the flip mirror used to couple the alignment laser into the system blocks one part of the

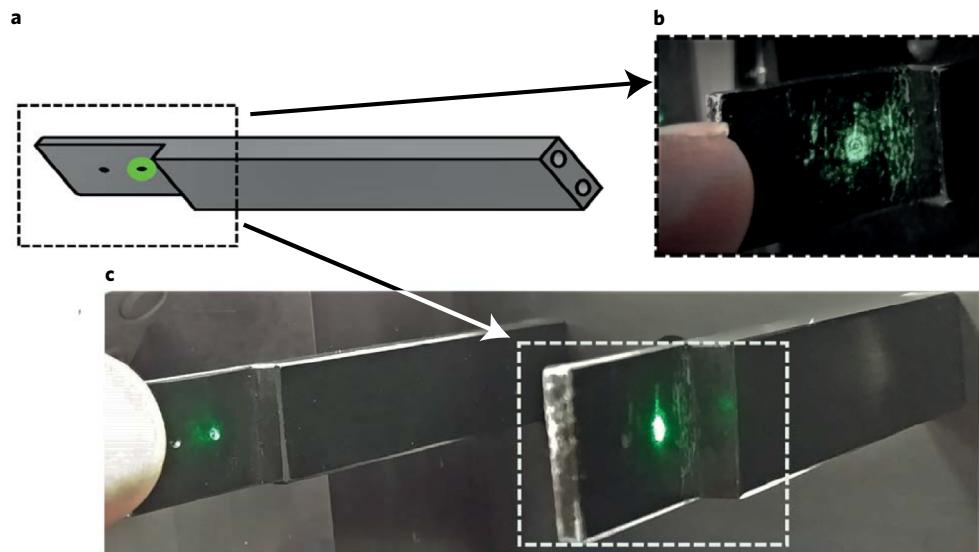


Fig. 3 | Detection path beam-alignment tool. Diagram (a) and a photo (b) of a pinhole alignment tool with a 3-mm 532 laser incident on the target pinhole when installed for alignment. Two alignment tools are used together for alignment of a straight beam path in each segment of the emission beam path (c).

image separation path from being directly accessed. Subsequently, the alignment laser traverses the upper portion of the image separation path and is then folded back into the lower portion, by removing the prism mirror M17 (Fig. 1a), for alignment in the opposite direction.

After the cavity, excitation and image separation paths have been aligned, a bead sample is mounted (Steps 142–153) onto the microscope for the first time and is imaged (Steps 154–186). The lower objective’s XY position and sample stage’s Z position are set (Steps 165–186). Once an image of the bead sample is located on the camera, the bead images are used to set the excitation and emission aperture sizes and position the four interference images on the camera (Steps 187–204). With a bead sample mounted and in focus, the critical step of locating interference for the first time is carried out (Steps 205–219). Next, the DMs are calibrated and centered (Steps 220–233). The interference phase across the field of view will require adjustment by iteratively moving the tip/tilt of the NPBS and the lower objective lens as covered in Steps 234–248. The phase relationship between the four interference images is calibrated by adjustment of the quartz wedge pair following Steps 249–273, and a system flat file for both DMs is generated following Steps 274–294. Data are collected from a bead sample to generate a transformation between the reference image and the three other interference images (Steps 295–302) and to calibrate the phase of the interference images (Steps 303–314). Additional optics are added for an objective lock (keeping the relative position of both objectives constant) in Steps 315–329. Next, beads are added to a cell sample, and the specimen is mounted on the microscope (Steps 330–342). Finally, image data are collected (Steps 343–347) and analyzed to generate a reconstructed image (Steps 348–356). Further overview information for specific subsections of the Procedure, with relevant step numbers, is presented below.

General beam alignment (Box 1)

To ease alignment, a pinhole alignment tool (custom part, FAB-OX0010), shown in Fig. 3, can be inserted into pre-set positions within the interference cavity and image separation path. Except for the tower assembly, two pre-set positions are placed in each straight section of the emission paths. Thus, using the general beam alignment procedure, described in Box 1, an alignment laser may be made collinear with the line defined by the pinhole centers. We recommend having at least four alignment tools on hand for convenience. In addition, in the image separation path, iris diaphragms are placed in predefined positions to assist with alignment.

Interference cavity alignment (Steps 12–68)

Alignment of the interference cavity is the most critical part of the system setup. As described in ‘Optical overview’ in the Introduction, fluorescence emission originates from the sample, is collected by two opposing objective lenses, propagates through the interference cavity upper and lower paths

Box 1 | General beam alignment procedure ● **Timing** 2–20 min

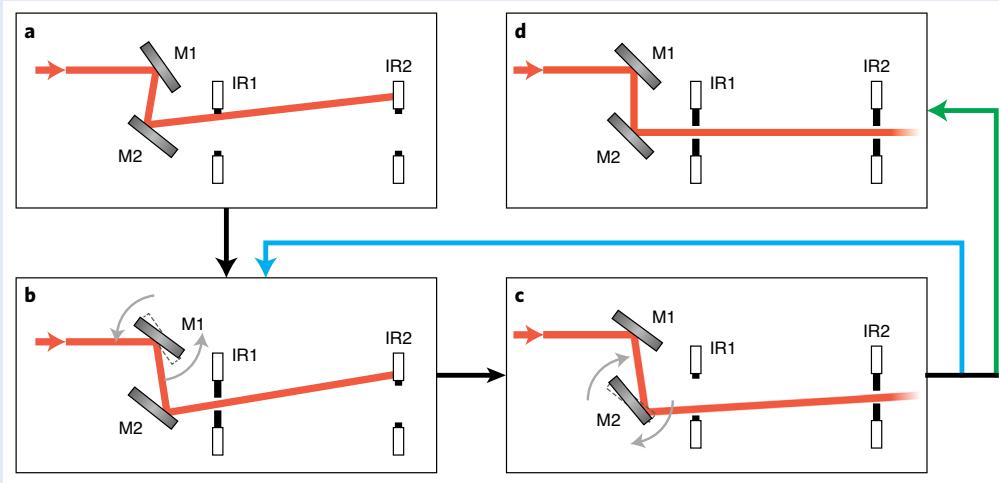
Throughout the procedure a series of pinholes and irises are used as markers for easy alignment. In many instances, the alignment laser must be adjusted to pass through two sequential pinholes (or irises), and in most cases a common procedure, probably already familiar to anyone experienced in optical alignment, is used to achieve this result.

By design, in straight sections of the interference cavity and image separation paths, there are two pinholes or irises whose fixed center positions define a line (optical path). These straight sections are connected by reflecting elements, such as mirrors, that are, in most cases, held by tip-tilt mounts. Here, the goal is to use the two adjustable elements preceding the pinhole or iris pair to colinearly align the alignment laser beam with the line defined by the pinhole or iris centers. This is accomplished using the procedure, as illustrated in the figure below. Initially, following from this figure, an incoming beam traverses two mirrors, M1 and M2, and passes two iris diaphragms, IR1 and IR2.

The procedure is as follows:

- 1 Ensure that irises IR1 and IR2 are initially fully open (see panel a of the figure).
- 2 Partially close IR1 while adjusting mirror M1 such that the center of the input beam hits the center of partially closed IR1 (see panel b of the figure). IR1 should be partially closed to make assessment of the beam position easier.
- 3 Fully open IR1 and partially close IR2 (see panel c of the figure) to make assessment of the input beam position on IR2 easier. Adjust mirror M2 until the center of the input beam hits the center of IR2.
- 4 Repeat steps 2 and 3 until the beam passes the center of both IR1 and IR2 (see panel d of the figure). This often requires many iterations.

The procedure is identical for pinholes except that a pinhole must be removed/inserted while an iris may be opened or closed.



(Fig. 1a) separately and is combined at the NPBS where interference occurs. Extreme care is needed during interference cavity alignment to ensure that the upper and lower paths produce negligible difference in image distortion and aberrations. The alignment procedure can be simplified by using two plastic 3D-printed shutter mounts for SH0 and SH1, in the CAD repository as 4pi-shutter-mount-V2.stl. These mounts are lower profile than the machined versions (currently part of the CAD model) and have sufficient flex to allow the insertion and removal of the pinhole alignment tool in positions PH1 and PH3 without removing the mount and shutter.

Excitation path alignment (Steps 69–101)

The excitation path is best set up when the alignment procedure reaches the two opposing objective lenses. After both objective lenses are installed, removing them to align the excitation path is inconvenient. The excitation path and emission path of the system are mounted onto the two opposite sides of the vertical breadboard at the center of the microscope as shown in Fig. 2. At the time of publication, the custom control software supports lasers from MPB Communications' visible fiber laser series or from Coherent's Obis series. Control for other lasers may be added to the software if desired. As such, we recommend purchasing 642- and 560-nm high power (>1 W) lasers from MPB Communications and lower-power 488- and 405-nm Obis lasers from Coherent. The excitation lasers chosen for the system should all be coupled into one single mode fiber. Light launched from the fiber is collimated by a reflective collimator (RC12FC-P01, Thorlabs) to minimize chromatic effects. After collimation, excitation light follows a straight path through a rectangular aperture and a lens that focuses the excitation light into the back aperture of OBJ1 (the lower objective lens). The rectangular aperture should be adjusted such that the excitation light illuminates an ~25 × 25 μm square area of the sample (this corresponds to an aperture opening of ~4.2 × 4.2 mm). The beam size, aperture size and focusing lens have been selected such that the sample is more ‘uniformly’ illuminated (~25% reduction in intensity from the center of the field of view to the edge). The trade-off, however, is that

most of the excitation light is ‘thrown away’ before reaching the sample. This, in combination with the high powers needed for SMS-based microscopy, necessitate high-power light sources. The excitation path also includes two flip mirrors that direct the excitation beam through an alternative path, resulting in a large illumination area at the sample (~100- μm diameter) for sample location and identification.

When high-power lasers are launched into a single-mode fiber, nonlinear effects may result in unexpected wavelengths being generated inside the fiber. This can result in an increased background on the data-collection camera. Thus, we advise having a laser clean-up filter on hand to be mounted after the fiber output. A good option, corresponding to the dichroic mirrors and emission filters installed in the cavity, is FF01-390/482/563/640-25 from Semrock. Be sure to mount all optics, optomechanics and other elements of the excitation path on the system before starting Step 69.

Image separation path alignment (Steps 102–141)

The image separation path starts at the NPBS and ends at the image acquisition camera. It is centered around the PBS, PBS0, where the s- and p-polarization channels are separated. The separated images are relayed onto the image acquisition camera where the four phase-shifted images are captured.

After alignment of the 4Pi cavity, two beams from the alignment laser (one transmitted from the NPBS and the other reflected) will transmit through both objective lenses and return to the NPBS, where they combine and exit the 4Pi cavity. However, one beam is blocked from entering the lower portion of the image separation path by the flip mirror (FM0) that is used to couple the alignment laser beam into the system. Because the lower portion of the image separation path is blocked, the alignment laser beam traverses the upper portion of the image separation path and is then folded back into the lower portion. Alignment in the opposite direction is facilitated by removing the prism mirror M17.

Sample mounting (Steps 142–153)

Two opposing objective lenses sharing a common focal plane preclude conventional sample mounting methods (slide, dishes and wells). The working distance (200 μm) of the objectives used in this system means that the available space between the two lenses is ~740 μm in total. As the sample must be viewed from two sides simultaneously, the sample must be sandwiched between two coverslips. Two no. 1.5 \times 25-mm round coverslips in a minimalist custom compact holder (FAB-P0035) are used for sample mounting. The two coverslips are held in place with two-component silicone adhesive. The procedure is identical for mounting bead and biological samples. Following Steps 144–153 should result in thicknesses between the coverslips of <50 μm .

Only coverslips with tight thickness tolerances should be used (e.g., coverslips with thickness between 165 and 175). Good options for no. 1.5 \times 25-mm round coverslips would be Thorlabs cat. no. CG15XH1 or Marienfeld cat. no. 0117650. Some imaging buffers slow the reaction time of the two-component silicone adhesive, requiring more time for the silicone adhesive to cure. Using unequal amounts of the two components will also have an adverse effect on the rigidity and cure time. Be sure to allow enough time for the silicone adhesive to cure before imaging.

Loading a sample and setting OBJ1’s lateral position (Steps 154–186)

The next section of the procedure describes how to load a mounted sample on the microscope for the first time. The upper objective (OBJ0) is fully retracted, and the mounted sample is placed on the sample stage. The three DC mike motors supporting the sample stage stack must be adjusted to coarsely bring the sample into focus with OBJ1. Once these motors have been adjusted, their position will remain constant when installing other samples. The sample is fully focused using the sample piezo Z stage, and the upper objective is engaged. The lateral position of OBJ1 is adjusted to merge the images from OBJ0 and OBJ1.

Initial interference location (Steps 205–219)

Interference will occur when the optical path length difference between the upper and lower cavity arms is less than the coherence length of a fluorescent emitter (typically between 5 and 10 μm). The path length difference between the two cavity arms is adjusted by translating the entire stage stack assembly (sample and both objective lenses) vertically. In practice, this is accomplished by synchronously moving the two translation stages holding the stage stack assembly (ASI, LS-50). During initial setup, it is possible that the stage stack will need to be moved vertically up or down by 1 or 2 mm to locate interference.

Locating interference for the first time is complicated by several factors. First, as the interference location is unknown, it is possible to move the stage in the wrong direction relative to the initial position. Second, there is a trade-off between how fast you move the LS-50 stages and how long interference will be observable. That is, if the LS-50 stages are moving at 1 $\mu\text{m}/\text{s}$, you will have 5 s to observe interference. However, if you need to scan over a large range to locate interference, a 1- $\mu\text{m}/\text{s}$ speed may be impractical. Third, while in motion, the LS-50 stages will not be perfectly smooth and synchronized. This typically manifests as lateral objective misalignment (vibration) when moving over larger distances (100 or 200 μm). Thus, while looking for interference, one must periodically realign the objectives.

With these considerations in mind, this section of the procedure describes how to initially locate interference. However, we encourage the builder to exercise good judgement during this process. For example, after the stage stack has been translated a reasonable distance without locating interference, the builder should decide if the scan direction was incorrect and return to the initial position or continue the current scan.

DM centering (Steps 220–233)

Once calibrated, the DM must be centered relative to the objective pupil plane. That is, the center of the DM must be imaged to the center of the objective lens pupil plane. Initially, the DMs are approximately centered based on the alignment laser beam during the interference cavity alignment. However, a more accurate centering is needed and will be carried out later in the procedure using fluorescence. The microscope requires application of astigmatism for imaging thick samples, and, subsequently, the DM must be correctly centered in the objective pupil for the calibrated mirror modes, found by using the slot in the interferometer, to be valid.

Phase adjustment across the field of view (Steps 234–248)

After interference is located, it is likely that the interference phase will vary across the field of view because of small tilts in the emission path. This can be observed as a fringe pattern when imaging a high-density bead sample. Removing phase variation across the field of view is required for appropriate image reconstruction. Subsequently, this section of the procedure describes how to remove the spatially varying phase across the field of view.

Quartz wedge calibration (Steps 249–273)

The 4Pi-SMS microscope uses a quartz wedge pair (one wedge bonded to a BK7 flat) in the upper cavity path in combination with a quartz flat (bonded to a BK7 wedge with another BK7 wedge free to translate) in the lower path to control the phase difference between Δs and Δp . That is, the quartz wedge controls the phase difference between the four images. In practice, each set of four images collected by the imaging camera represents four interferometric images (measurements) with known phase shift collected in a single shot. The quartz wedge pair (W0) in the upper cavity path must be positioned to give a $\pi/2$ phase shift between the four images collected on the imaging camera. Although a quarter waveplate could be used in place of a wedge pair/flat combination, it would eliminate the possibility of easily using spectrally separated fluorophores. Calibrating the quartz wedge position requires multicolor fluorescent beads prepared as described in ‘Reagent setup’. The multicolor beads are typically less ‘sticky’ with glass, and a higher concentration (1:100 (vol:vol) from stock concentration) should be used.

DM system flat and astigmatism calibration (Steps 274–294)

The PSF of the instrument should be the same in all four imaging channels once the image separation path is well aligned. The PSFs obtained by the two objective lenses should also be the same. If the PSFs are not reasonably close, it is useful to optimize them using the two DMs by generating a so-called ‘system flat’ (compensating for any system-induced aberrations) following this section of the procedure. Generating a system flat is also important as the DMs will be used to apply astigmatism to the PSF when imaging biological samples, and starting with an aberration-free PSF is necessary for correct application of the astigmatism mode.

Image registration and phase calibration (Steps 295–314)

Before imaging any biological samples, calibration data must be collected for three purposes: (i) to create a transformation that allows three of the images to be mapped onto the reference image; (ii) to ascertain the phase delay between two of the four images, S1 and P2; and (iii) to determine the

calibration between the phase and astigmatism for accurate axial localization. The calibration information needed for this is generated from two bead images. In brief, a bead sample is mounted on the microscope, and a single frame from either OBJ0 or OBJ1 is collected (Steps 295–302). This single frame image is used to generate the transformation that maps three of the images onto the fourth reference image. Next, a Z-stack is collected with interference ‘on’ (both objectives imaging, laterally aligned, and the cavity adjusted for maximum interference) and astigmatism applied (Steps 303–314). This is used to find the relationship between the phase and Z-position as determined by astigmatism and enables the determination of Z position over a depth of greater than $\lambda/2$.

Data analysis (Steps 348–356)

Once the single frame image and Z-stack are collected as described above, calibration files are generated using the MATLAB analysis software. Further steps on how to analyze biological image data with these calibration files are described in this section of the procedure.

Experimental design

Personnel

For successful planning, parts acquisition, building and troubleshooting, no fewer than one full-time, experienced microscope builder should be employed.

Laboratory recommendations

As the mechanism used in a 4Pi microscope to enhance axial resolution is light interference, the necessity of a quiet, temperature-controlled, and vibration-free environment is critical for maximum performance. As such, we strongly recommend the careful selection of the laboratory space where the microscope will be housed. Ideally, the laboratory will be on the ground level or basement and without windows. Furthermore, the space selected must be located away from mechanical and acoustic vibration sources such as lifts/elevators, air handling units or freezer banks. Thermal stability is also important. We recommend a room capable of maintaining a temperature within ± 0.5 °C, including overnight and weekends, as the large heat capacity of the instrument and optical table lead to slow response times relative to ambient temperature changes. Air conditioning systems should not blow air directly onto the system, to minimize mechanical vibrations leading to fluctuations in the interference pattern. The option of temporarily disabling any climate control systems that blow air around the system is recommended. The selected laboratory space should be large enough for an optical table no smaller than 1,200 × 900 mm with personnel access to the table from at least three sides.

Of the existing systems, some groups have located their excitation lasers on the same optical table as the microscope. However, this requires a larger optical table. Alternatively, excitation lasers may be located on a different bread board, next to or underneath the microscope table, or in a different room, and fiber-coupled to the microscope.

It is important to note that the microscope presented here includes many devices with separate controllers and power supplies, some of which include cooling fans (vibration risk) and relatively short cords. These should not be placed on the optical table with the microscope. We strongly recommend that builders carefully consider where to place controllers and power supplies (a ceiling-mounted overhead equipment rack and a 19-inch rack system are both good options) before assembling the instrument and ensure that sufficient laboratory space is available.

Purchasing and delivery

We strongly recommend that a pragmatic approach is taken when planning the required time for following this protocol. Significant time (1–2 months) will be needed to collect quotations and place orders. The parts list presented here is for materials sourced from within the United Kingdom, and alternative vendors or distributors may need to be located for your geographic region. Furthermore, several key items (e.g., the magnification-matched objective lenses, the DMs and the piezo stages) have long delivery times. Specifically, this includes a pair of magnification-matched objectives (Olympus, UPLSAPO 100XS), a pair of DMs (Boston Micromachines, Multi-DM 5.5), several piezo stages (PI) and two long-travel linear stages (Applied Scientific Instrumentation, LS-50). Delivery times up to 6 months for individual items should be expected.

Objective lenses with closely matched magnifications (within 0.3%) must be used to ensure image overlap across the field of view. If possible, have objective lenses with matched magnifications selected

by the vendor. Alternatively, it may be necessary to purchase three or four lenses and use the closest matched set (it may be possible to return the unused lenses or make other arrangements with your local supplier).

Optical table selection

In the selected laboratory space, an optical table no smaller than 1,200 × 900 mm should be installed. Table legs that provide active vibration isolation are strongly recommended (e.g., PFA52507, Thorlabs). Once the chosen optical table has been installed, ensure that it is level and that the active vibration-isolation legs are functioning correctly with adequate range of motion. Careful consideration should be given to the placement of excitation lasers. Of the existing systems, some groups have located their excitation lasers on the optical table with the microscope. However, this requires a larger optical table. If space constraints limit optical table size, excitation lasers may be located on a different breadboard.

It is important to note that the microscope presented here includes many devices with separate controllers and power supplies, some of which include cooling fans (vibration risk) and relatively short chords. These should not be placed on the optical table with the microscope. We strongly recommend that careful consideration is given to where the controllers and power supplies will be located (overhead equipment racks or a 19-inch rack system are good options).

Machine shop selection

The system described here includes many custom parts that require high-precision machining and anodization. To date, all 4Pi-SMS systems built have all used commercial shops not connected with any universities. However, a well-equipped computerized numerical control facility with trained machinists should be capable of producing the required parts regardless of whether internal or external to a university. With the volume of parts required, it is inevitable that some errors will be made. For this reason, choosing a nearby machine shop is ideal to reduce logistics when returning parts for rework. Selecting a machine shop that can have parts anodized is also a key requirement. As machining quality may vary from shop to shop, as with any large purchasing decision, we recommend checking references and getting a minimum of three quotes before placing an order.

Alignment dowel pins

Throughout the microscope, alignment pins are used when connecting various mounts with their respective breadboards. The custom part drawings contained within the online CAD repository (<https://github.com/4Pi-SMS-consortium/CAD-files>) specifically call out mechanical tolerances for holes that the alignment pins should fit into. As several components must be removed during alignment, the use of interference fits is undesirable: placement of alignment components at their constrained locations should be possible by hand pressure only. We have therefore specified a transition fit for dowel pins into the alignment mounts. Despite best efforts, anodization will result in some undersized dowel holes requiring (i) pressing the pin in with high external force, (ii) re-machining or (iii) enlarging with a reamer by hand. We strongly encourage builders to check all pin hole locations and fits as parts arrive to reduce delays. Purchasing extra pins with large undersized tolerance is also advised as an extra precaution.

Laser safety

The 4Pi-SMS microscope, as presented here, uses high-power lasers (up to 2 W), which pose a significant health risk. These systems should be built only in consultation with local laser safety personnel and rules. Furthermore, systems should be fully enclosed, and lasers should be interlocked on either the enclosure or the laboratory door to prevent accidental exposure. During setup and alignment, additional care must be taken, as there will be open beam paths with lasers pointing in unexpected directions.

Alignment laser guidelines

The alignment strategy used in this protocol is to use a small laser that acts as a proxy for fluorescence emission. However, as it is impractical to place an alignment laser at the sample plane, an alignment laser is set up on the horizontal optical table and coupled into the system using a flip mirror. The alignment laser initially traverses the cavity, opposite to the emission propagation direction, before passing through both objectives, back through the cavity and into the image separation path. The flip mirror, FM0, allows the alignment laser to be coupled into the system and, later, allows fluorescence

to be directed out of the microscope if a larger field of view is needed on a second camera to ease sample location. Many suitable lasers exist that may be used for alignment. The key features needed are (i) ~3–5-mm beam diameter and (ii) low enough power to be safely used in an open beam setting (refer to your local rules for acceptable levels).

One convenient option is a 532-nm alignment laser from Thorlabs (part number CPS532). We also recommend using a variable neutral-density filter to adjust the power during alignment. Mounting the laser in a kinematic tip/tilt mount may also be a convenient option. The alignment laser setup, as detailed in ‘Experimental design’, is not currently part of the CAD model but can be easily reproduced with common stock parts.

Alignment camera

Several steps in the procedure use an alignment camera to visualize the beam, or optical components relative to the centre of the beam. For example, while adjusting the axial DM position, a camera is positioned in one of the objective lens mounts. Thus, having a compact universal serial bus (USB) camera with a known sensor location, relative to its mounting body, is critical. Although many cameras exist that may be used for this purpose, here we recommend two equivalent options along with a specific mounting recommendation. The two recommended cameras are: (i) Edmund Optics, part number 84-933 (complementary metal-oxide semiconductor (CMOS) monochrome USB camera, board level) and (ii) Thorlabs, part number DCC1545M (USB CMOS camera, mounted).

In both instances, the cameras are sourced from a common supplier (IDS Imaging), and a similar camera may be sourced directly from IDS. The camera provided by Thorlabs is in a large plastic housing that may be removed. Both cameras integrate onto a minimal compact camera mount directly compatible with Thorlabs’ SM1 thread. This camera mount is found in the CAD repository under the ‘Pupil Plane Camera’ directory. Mounting the camera on this custom mount ensures that the sensor is mechanically centered and that the sensor surface is a known distance relative to the mounting flange (necessary for positioning the sensor at specific axial positions during alignment). For camera control, the best option is installing drivers and a camera manager directly from IDS Imaging (this also allows the camera to be used with the custom microscope control software). This software package is named ‘uEye’ by IDS. At the time of publication, this software may be located by simply searching for ‘ids ueye download’ in your preferred search engine. Please note that the ‘uEye’ software package will conflict with the popular ‘ThorCam’ package from Thorlabs. Although both may be installed on the same computer at the same time, only one will work. In addition, the cameras listed above often fail to connect when plugged into a USB hub, and a direct connection to the computer is recommended.

Microscope enclosure

After setup of the 4Pi-SMS, it is critical to set up an enclosure around the system to minimize stray light incursion and vibration due to air movement (e.g., due to an air handling system). Take care to position the microscope and electronic cables on the optical table, leaving enough space to enclose the microscope later. As the specific enclosure design depends on the size of the optics table, where device controllers have been located and the preferences of the builder, we have omitted any plans as part of this protocol.

Computer infrastructure and data storage

Although it is possible to acquire and analyze data on the same computer, to date, all existing systems have separated these tasks between two computers: one for data acquisition and one for data analysis. The high bandwidth of scientific CMOS (sCMOS) cameras makes it possible to rapidly obtain large data sets. At ~700 kB/frame after cropping, datasets from one field of view are ~100–200 GB in size, such that a typical day of imaging can be contained on a single fast hard drive (e.g., a 3-TB PCIe solid-state drive (SSD)). More inexpensive hard-disk drives (HDDs), now available with ~15 TB/drive, are suitable for post-acquisition storage. The instrument control computer itself, if equipped with an appropriate SSD and HDD, is therefore adequate during early and intermediate stages of use. Additional solutions in the form of compression and distributed storage, either specific to localization microscopy data¹³ or more general, may be helpful as the user base of the instrument grows.

Sample index matching

Successful imaging has been carried out using a standard water-based imaging buffer for dyes Alexa Fluor 647 and CF660C^{4,9}. However, matching the sample refractive index (RI) will reduce the

likelihood that adjusting the sample focus will detune the interference cavity, away from maximum interference, which may compromise axial resolution. Furthermore, to preserve high-resolution imaging throughout an entire cellular volume, up to 10 μm in depth, the RI mismatch between the samples, the mounting media and the immersion media should be minimized¹⁴. In addition, in most cases when imaging a biological sample with two opposing objective lenses, one objective lens will necessarily image through a longer optical path in the sample to reach the common focal plane. Subsequently, RI mismatch will result in differing amounts of spherical aberration between the two lenses. Several techniques are used to minimize this effect. Here, we use silicone immersion lenses with an immersion oil RI of 1.406 (Olympus, SIL300CS-30CC), close to that of bulk cellular material (1.33–1.38). Samples are mounted either in a standard water-based imaging buffer (~1.33) or an RI-matched imaging buffer (~1.4). We successfully tested three different RI-matched dSTORM imaging buffers. These contained concentrations of 1,200 U/ml catalase, 100 U/ml glucose oxidase and 35 mM cysteamine (MEA). Finally, residual spherical aberration is compensated by the DMs in each cavity path¹⁵. We encourage the reader to evaluate different options (i.e., water-based imaging buffer or different RI-matched buffers) for their application. Commercial dSTORM buffer options are also available.

Multi-color imaging

Multi-color imaging can be achieved using dyes with well-separated spectra and recorded sequentially for biological structures close to the coverslip^{4,16,17}. However, the recently developed salvaged fluorescence approach has introduced multi-color imaging with minimal crosstalk and negligible chromatic aberrations into 4Pi-SMS and is the preferred method for multi-color imaging⁹. The 4Pi-SMS microscope proposed here can be upgraded for salvaged fluorescence imaging with minor configuration changes on the excitation path (no changes to the detection path are needed). In addition, there are ongoing development efforts across multiple institutions, and we encourage readers to reach out for advice regarding best practices for multi-color imaging and any other questions.

Automatic objective alignment and tracking

As detailed in ‘Conceptual description of the 4Pi-SMS setup’ in the Introduction, in a 4Pi geometry a sample is imaged from opposing sides simultaneously. Subsequently, XY objective alignment is critical for lateral image alignment. That is, if the objectives are misaligned laterally, the resulting images will not laterally overlap, and interference cannot take place. Similarly, maintaining a set Z separation between both objectives is critical for ensuring there is a common focal plane. Furthermore, axial objective drift may compromise interference and, subsequently, axial resolution. Thus, to maintain XY and Z objective alignment while imaging, an active tracking system is used. Specifically, the system uses an NIR laser (940 nm) propagated through the two objectives in the opposite direction to the fluorescence excitation beam. The 940-nm beam is separated from the illumination path by a dichroic mirror, astigmatism is deliberately added and the astigmatic spot is imaged onto a camera. Tracking the position, size and asymmetry of the astigmatic spot allows closed-loop XY and Z control of relative objective positions via the objective piezo stages (P-612.2SL and N-565.260).

Data analysis

Appropriate data analysis for 3D image reconstruction is a critical part of the 4Pi-SMS microscope protocol presented here. The custom, MATLAB-based analysis package has evolved over several years and continues to be developed^{4,18,19}. The importance of properly implementing the analysis package is as important as building and aligning the instrument. The full details of the analysis package have been previously described⁴ and will be omitted here. Rather, we briefly describe the general procedure in the paragraphs below.

To begin, two calibration files are generated. First, a 2D affine transformation file that contains the parameters for each of the four images to be mapped onto the reference image (phase image 1 by default) is generated. Mechanical drift caused by temperature changes or different positioning of the stage stack can perturb the microscope, and this calibration file should be generated daily. Second, a phase-calibration file that contains the phase shift between the s- and p-polarization channels is generated. This file is generated from a bead Z-stack. Typically, the phase shift between the images remains relatively constant from day to day, and this calibration needs to be performed only occasionally. Next, the four images in each camera frame are summed after registration. Single-molecule detection and fitting are performed on the graphics processing unit (GPU) to estimate the XY positions, standard deviations (σ_x, σ_y) and other information (photon number, background photon counts, log-likelihood ratios, etc.) of the molecules. The phase information (Z position) is estimated

from the zero-order reduced moment^{2,4} of the four phase images using the calibrated phase shift. A ridge-finding algorithm⁴ is used to unwrap the phase and convert it to an axial position using a wavelength-dependent modulation frequency. Drift correction is applied using a redundancy-based drift-correction method^{20,21} to correct sample drift in 3D. For thick samples requiring Z-stacks, 3D cross-correlation is used to align consecutive optical sections via overlapping information between adjacent optical sections. Finally, localization information is saved as comma-separated values in a text file for later analysis and visualization.

The analysis software and example data can be downloaded from the following links with instructions and expected results: <https://github.com/4Pi-SMS-consortium/4Pi-SMS-analysis> (analysis software) and <https://zenodo.org/record/3929647#.X6DB-INKiu4> and <https://zenodo.org/record/4022827#.X6DCL1NKiu4> (example data).

Visualization and post-localization analysis

Raw camera frames from the microscope may be opened and/or viewed with HCImageLive (Hamamatsu), Super-resolution Microscopy Analysis Platform (SMAP, <http://github.com/jries/SMAP>)²² or the PYthon Microscopy Environment (PYME, <http://python-microscopy.org/>). Although 4Pi localizations can be readily plotted in many software packages or custom code, specialized tools are recommended for viewing 4Pi localization data, rendering these data into super-resolution images in 3D and post-localization analysis. We recommend the following software packages. Vutara SRX, a commercial software package available from Bruker (see ‘Competing interests’), may be used to visualize 3D image data directly from the analysis software and provides conventional post-processing data analysis functionality (www.bruker.com/products/fluorescence-microscopes/vutara-super-resolution-microscopy/overview/srx-software-vutara-super-resolution.html). SMAP is a MATLAB-based, open source, image analysis and visualization software package that can read 4Pi-SMS localization ‘.csv’ files, includes 2D and 3D rendering capability and provides conventional post-processing options such as drift correction, filtering and analyses of localization statistics (<http://github.com/jries/SMAP>). PYME is a Python-based, open source, data collection, data simulation and image analysis software package that can read 4Pi-SMS localization ‘.mat’ files for visualization with the VisGUI app (<http://python-microscopy.org/>).

Materials

Reagents

- ! CAUTION** All reagents can be potentially hazardous and should be handled by trained personnel.
- Reagents required for preparing the biological test sample of interest (see ‘Biological test samples’ in ‘Reagent setup’ and Supplementary Methods)
 - Nanopure water (Thermo Fisher Scientific, cat. no. 10977035)
 - Glycerol (Sigma-Aldrich, cat. no. G5516-500ML)
 - Sodium chloride (Thermo Fisher Scientific, cat. no. AM9759) **! CAUTION** Sodium chloride may cause skin and eye irritation. Handle in a fume hood with appropriate personal protective equipment.
 - Tris, pH 8.0, 1 M (Thermo Fisher Scientific, cat. no. AM9856) **! CAUTION** Tris can cause skin and eye irritation. Handle in a fume hood with appropriate personal protective equipment.
 - Catalase (Sigma-Aldrich, cat. no. C40-100MG)
 - Glucose (Thermo Fisher Scientific, cat. no. 15023021)
 - β -Mercaptoethanol (Sigma-Aldrich, cat. no. M3148-25ML) **! CAUTION** β -Mercaptoethanol is toxic and dangerous for the environment. Avoid inhalation, ingestion and contact with skin and handle using appropriate safety equipment and measures.
 - 2,2'-thiodiethanol (TDE, Sigma-Aldrich, cat. no. 166782) **! CAUTION** TDE is an eye irritant. Handle in a fume hood with appropriate personal protective equipment.
 - MEA (Sigma-Aldrich, cat. no. 30070) **! CAUTION** MEA is a hazardous, acidic powder, and it should be used in a fume hood and be handled only by a professional wearing goggles, a dust mask, gloves and a laboratory coat.
 - 100-nm crimson beads (625/645 nm; Thermo Fisher, custom order) **▲ CRITICAL** The 100-nm crimson beads are the best option for bead sample, as they are bright and less prone to photobleaching. Alternatives are (i) 40-nm-diameter dark red beads (660/680 nm; Thermo Fisher, cat. no. F8789) or (ii) 100-nm-diameter red (580/605 nm) beads (Thermo Fisher, cat. no. F8801)
 - TetraSpeck fluorescent microspheres, 0.1 μ m (Thermo Fisher Scientific, cat. no. T7279)

- High-quality coverslips, Ø25 mm, no. 1.5 (Thorlabs, cat. no. CG15XH/Marienfeld, cat. no. 0117650)
- TwinSil sealant (Picodent, cat. no. 1300 1000)

Equipment

Optomechanical components

The complete system is composed of >200 off-the-shelf optics and optomechanical parts alongside dozens of custom machined parts. A complete parts list is available in Supplementary Table 1, and the latest parts list can be downloaded from <https://github.com/4Pi-SMS-consortium/CAD-files/blob/master/PartsList.xlsx>. The parts list at the time of publication is included as Supplementary Table 1. The current snapshot of the 3D microscope model (Autodesk Inventor) can be downloaded from the repository at <https://github.com/4Pi-SMS-consortium/CAD-files>.

Microscope control software

- NI LabVIEW 2016 64-bit
- NI Device Drivers
- NI VISA
- NI Vision Development Module
- Hamamatsu DCAMAPI
- Hamamatsu HCImage Live for acquisition camera control (<https://hcimage.com/download/>)
- IDS Imaging, uEye
- ASI, ASIConsole
- Thorlabs, Kinesis
- PI, PIMikroMove

Data analysis software

- MATLAB 2016 or later, 64-bit
- Latest Nvidia graphics driver
- CUDA Toolkit 4.2.9 or later, 64-bit (www.developer.nvidia.com)
- DIPImage, 64-bit

Image acquisition computer

▲ CRITICAL Many computer configurations may work for microscope control and data acquisition. At the time of publication, we used a computer with the following specifications for all data acquisition in this protocol: Dell Precision Tower 7910 Workstation, Windows 10 64-bit, Dual Intel Xeon processor E5-2609 v4 @ 1.70 GHz, 32 GB RAM, Samsung SSD hard drive PM87 SCSI Disk Device. General guidelines are as follows:

- Operating system (OS): Windows 10, 64-bit
- Central processing unit (CPU): quad-core CPU
- Random access memory (RAM): 16 GB minimum
- GPU: compute unified device architecture (CUDA)-compatible graphics card
- Hard drive: SSD for acquisition, normal HDD for data storage
- Motherboard: three open peripheral component interconnect express (PCIe) slots for one to two camera controller cards and a data acquisition (DAQ) card **▲ CRITICAL** Furthermore, we recommend installing the computer operating system on a SSD. SSDs in the control computer are also recommended for data capture to minimize delays or data loss at the high data rates. Conventional hard drives are not fast enough to sustain the up to 1 GB/s data rate but are sufficient for lower (<50 Hz) imaging rates. Once data have been collected, users can manually or automatically transfer raw images to centralized high-capacity disk arrays for longer term storage and/or analysis.

Image analysis computer

▲ CRITICAL Many computer configurations will work for 4Pi-SMS data analysis. At the time of publication, we used a computer with the following specifications for all image analysis in this protocol: Dell Precision Tower 7910 Workstation, Windows 10 64-bit, Dual Intel Xeon processor E5-2609 v4 @ 1.70 GHz, 32 GB RAM, Samsung SSD hard drive PM87 SCSI Disk Device, NVIDIA Quadro 6000 GPU. General guidelines are as follows:

- OS: Windows 10, 64-bit
- CPU: quad-core CPU

- RAM: 32 GB
- GPU: CUDA-compatible graphics card **▲CRITICAL** An NVIDIA GPU is essential, as the analysis software requires running the CUDA toolkit.
- Hard drive: SSD for OS installation and 10-TB normal HDD for data storage

Reagent setup

Enzyme stock suspension solution

Prepare 1 ml of enzyme stock suspension solution by mixing 429.3 μ l of nanopure water, 500 μ l of glycerol, 50 μ l of 1 M NaCl, 20 μ l of 1 M Tris (pH 8.0) and 0.7 μ l of β -mercaptoethanol. **▲CRITICAL** Prepare this solution freshly each time.

Catalase stock solution

Prepare catalase stock from enzyme stock suspension buffer containing 500,000 U/ml catalase. Aliquots can be stored at -20°C for several weeks. **▲CRITICAL** Contact the supplier for accurate values of active units per milligrams of protein and percentage of active protein.

Glucose oxidase stock solution

Prepare glucose oxidase stock from enzyme stock suspension buffer containing 13,500 U/ml. Aliquots can be stored at -20°C for several weeks. **▲CRITICAL** You may need to contact the supplier for accurate actual activity values.

dSTORM imaging base buffer

Prepare dSTORM base buffer by mixing 1.95 ml of nanopure water, 2.5 ml of 20% (wt/vol) glucose, 250 μ l of 1 M NaCl and 250 μ l of 1 M Tris-HCl at pH 8.0. Prepare fresh base buffer before imaging buffer preparation.

Water-based imaging buffer

Mix 978 μ l of dSTORM base buffer with 2 μ l of catalase stock, 10 μ l of glucose oxidase stock and 10 μ l of β -mercaptoethanol (Sigma-Aldrich, M3148-25ML). Prepare fresh buffer for each imaging session.

Enzyme stock solution for RI matching

Prepare 1 ml of enzyme stock solution in water that contains 24,000 U of catalase (C3155, Sigma-Aldrich), 2,000 U of glucose oxidase (G7121, Sigma-Aldrich), 85% (vol/vol) glycerol and 50 mM Tris-HCl (pH 8.0). Aliquots of the enzyme stock solution can be stored for several weeks at -20°C .

RI-matched imaging buffer

Prepare 126 μ l of 4 \times GLOX buffer by mixing 71.5 μ l of 70 % (wt/vol) glucose, 25 μ l of 1 M Tris-HCl (pH 8.0), 25 μ l of enzyme stock, 1 μ l of 1 M NaCl and 3.5 μ l of 5 M MEA. The three different RI-matched dSTORM imaging buffers are prepared in water and contain one of the following:

- 46.75% (vol/vol) glycerol and 25% (vol/vol) 4 \times GLOX buffer (RI: 1.4012)
- 38% (vol/vol) TDE (166782, Sigma-Aldrich) and 25% (vol/vol) 4 \times GLOX buffer (RI: 1.4055)
- 48% (wt/vol) sucrose (S0389, Sigma-Aldrich) and 25% (vol/vol) 4 \times GLOX buffer (RI: 1.4033)

The RI-matched imaging buffers should be freshly prepared.

RI-matched bead imaging buffer

Mix 370 μ l of TDE (Sigma-Aldrich, cat. no. 166782) with 630 μ l of 16 mM MgCl₂ (105833, Merck) in water. The final medium has 10 mM MgCl₂ and an RI of 1.403. The RI-matched buffer can be stored at 4 $^{\circ}\text{C}$ for 6 months.

Bead samples

Fluorescent bead samples with different densities are required during the instrument alignment. Start bead sample preparation by diluting bead stock from 1:10,000 (vol/vol) for a sparse sample up to 1:100 (vol:vol) for a dense sample in RI-matched bead imaging buffer. Place the first coverslip in the sample holder and the second coverslip on a clean surface where it is easy to access with forceps. Put 5 μ l of RI-matched bead medium on the second coverslip and 5 μ l of the diluted beads on the first coverslip. Place the second coverslip upside down on top of the first coverslip in the sample holder.

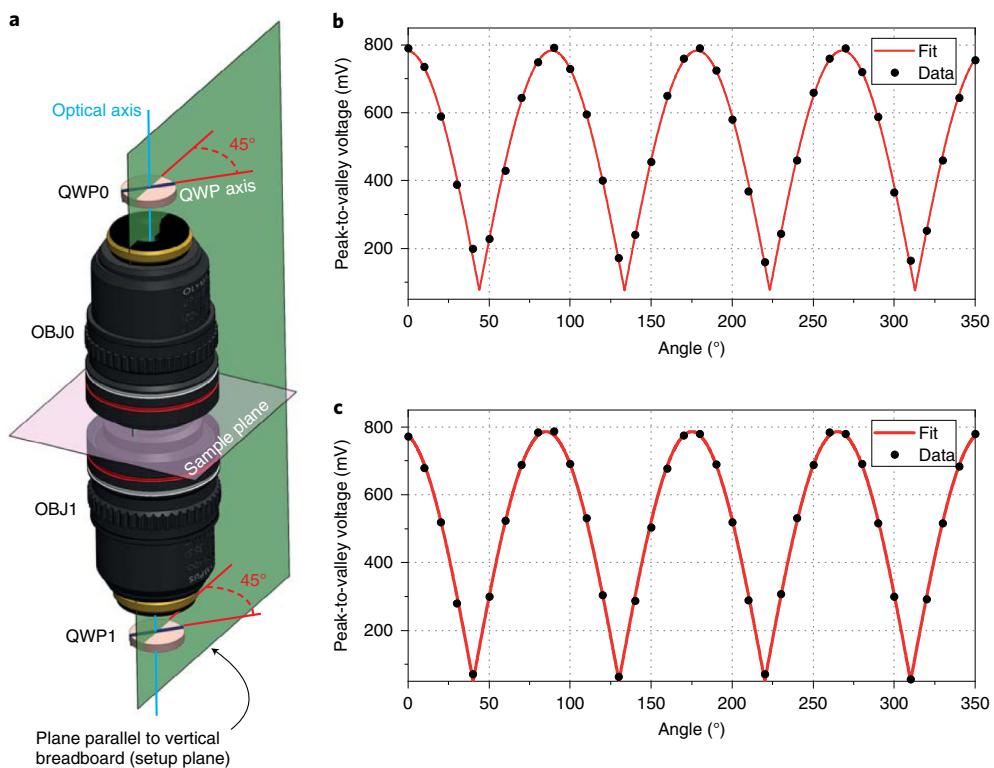


Fig. 4 | Waveplate orientation and example calibration data. QWP orientation in the microscope (**a**) and example wave plate orientation calibration data for both wave plates (**b** and **c**).

Carefully blot around the edges of the coverslip with a lint-free, absorbent tissue to remove the excess liquid. Seal the coverslips in the sample holder using TwinSil sealant (see details in Steps 150 and 151). The sample will be ready to mount after 15 min, when the sealant is set. Bead samples may be safely stored for 2 months at 4 °C.

Biological test samples

Microtubules or nuclear pore complexes are used to test the imaging performance of the system (see Supplementary Information for details). For microtubule sample preparation⁹, follow the methods section, subsection ‘Microtubule samples’, in Zhang et al.⁹ using no. 1.5 Ø25-mm round coverslips. For preparation of the nuclear pore complex samples²³, follow the methods section, subsection ‘SNAP-tag labelling of fixed cells’, in Thevathasan et al.²³ using no. 1.5 Ø25-mm round coverslips. The sample should be freshly prepared for each imaging session.

Equipment setup

▲ CRITICAL Before starting the procedure, we recommend that each computer-controlled component is tested with manufacturer-supplied software to ensure correct operation. Ideally, this is carried out as devices arrive to ensure anything damaged during shipping is identified and returned for repair before it is installed in the system. Exchanging a defective device may be difficult depending on its location within the microscope.

QWP setup

The 4Pi-SMS microscope includes QWPs: one positioned as the first optical element above the upper objective (OBJ0/QWP0, Fig. 1) and the second as the first optical element below the lower objective (OBJ1/QWP1, Fig. 1). The purpose of the QWPs is to split the linearly polarized (random orientation) fluorescent emission into equal intensities along the s- and p-polarization directions (channels). To maximize interference contrast, and for proper microscope operation, the orientation of the two QWPs must be carefully set. Overall, the QWPs should be oriented so the fast and slow axes are at 45° in or out from the vertical breadboard as presented in Fig. 4a. Both waveplates should have their fast/slow axes aligned with each other. To begin, mount both wave plates (AQWP05M-600, Thorlabs)

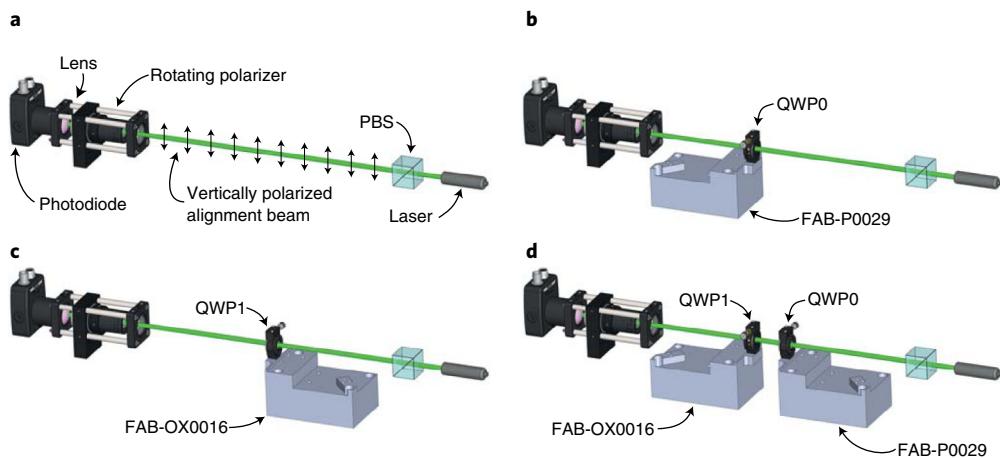


Fig. 5 | QWP calibration setup. **a**, QWP calibration is begun by directing a vertically polarized alignment laser into a continuously rotating polarizer with photodiode. **b**, QWPO is inserted into the beam, and calibration data are collected. **c**, QWPO is removed, QWP1 is installed and calibration data are collected. **d**, Both QWPs are installed to finalize their orientation.

in their respective holders (PRM05/M, Thorlabs) and attach these holders to their respective locations on parts FAB-P0029 and FAB-OX0016 (Filter Cube Holder Upper and Lower, respectively) in the correct orientation (fine adjustment knob in the correct direction; see the CAD model or Fig. 5). Please note that the QWPs are mounted in one-inch-diameter holders and must be removed from these holders before being mounted in the PRM05 rotation mounts. Waveplate alignment is performed outside of the 4Pi system on a standard optical table or breadboard and is best done before building the system. In addition to the mounted waveplates, an alignment laser, polarizer or PBS cube and a polarization-checking tool (Fig. 5) are needed. For the polarization-checking tool, we use a photodiode with a rotating polarizer (G06511700 from Qioptiq Photonics in combination with GTH10M-A/B from Thorlabs); having a commercial polarization analyser (e.g., Schäfter + Kirchhoff, SK010PA) may make the process simpler, but here we give the procedure for wave plate alignment using the more accessible photodiode and polarizers.

- 1 Mount the QWPs in Ø1-inch holders. Remove both wave plates from these holders by unscrewing their respective retaining rings.
- 2 Mount each waveplate into a Ø0.5-inch rotation mount (PRM05/M, Thorlabs).
- 3 Connect one of the PRM05/M rotation mounts onto part FAB-P0029 and ensure that the orientation is correct (fine adjustment knob in the correct direction; see the CAD model (https://github.com/4Pi-SMS-consortium/CAD-files/blob/master/4Pi-Oxford-Packed/Workspaces/Workspace/4Pi-Oxford/_TOP_4Pi-Oxford.iam) or Fig. 5).
- 4 Connect the remaining PRM05/M rotation mount onto part FAB-OX0016 and ensure that the orientation is correct as in the previous step.
- 5 Set up an alignment laser such that its beam is straight along the holes of the optical table or breadboard with height equal to a polarization-checking tool as presented in Fig. 5a. Ensure that the alignment laser beam is parallel to the surface of the table.
- 6 Place a PBS or polarizer in the alignment laser beam path such that the resulting polarization is perpendicular (vertical) with respect to the optical table or breadboard surface as presented in Fig. 5a.
- 7 With the linearly polarized laser beam directed into the polarization-checking tool, connect the photodiode of the polarization-checking tool to an oscilloscope (o-scope). As the polarizer in the polarization-checking tool continuously rotates, the linearly polarized laser beam intensity on the photodiode will oscillate between a minimum (close to zero) and maximum (full transmission) value resulting in a sinusoidal voltage on the o-scope.
- 8 Place QWP0, mounted to FAB-P0029, onto the optical table such that the polarized laser beam passes through the center of the waveplate and the light propagation direction is perpendicular to the waveplate surface as presented in Fig. 5b. Take care to place the polarization-checking tool into the beam path after QWP0, leaving enough room to add QWP1, mounted to FAB-OX0016, into the beam path without having to move any existing components.

- 9 Move QWP0 to the zero-degree mark on the rotation mount scale setting and record the peak-to-valley value (amplitude) from the photodiode as measured on the o-scope.
- 10 Move QWP0 to the 10-degree mark and record the peak-to-valley value on the o-scope. Repeat this process with 10-degree increments for one full rotation; at each position, record the peak-to-valley value from the o-scope. This gives a data set for peak-to-valley intensity versus angle for QWP0 (Fig. 4b).
- 11 Fit the intensity versus angle data with a modified sine function to find the minimum intensity angles as presented in Fig. 4b. Here, intensity versus angle data are fitted with $y(x) = A * \sin(mod((x - \emptyset)/(period/\pi), \pi)) + offset$. The four peak-to-valley intensity minimums correspond to the waveplate angles that result in circular polarization, when the fast and slow axes are at 45° with respect to the vertically polarized alignment laser. Record the four angles that produce the intensity minimums (circularly polarized light).
- 12 Remove QWP0 and insert QWP1 as shown in Fig. 5c. Repeat the measurement of intensity versus angle for QWP1 and record the values. Fit the intensity versus angle data for QWP1 and record the four angles that produce an intensity minimum (best circular polarization) as presented in Fig. 4c. In this way, for each waveplate, the angles at which the fast/slow axes are at 45° with respect to the input polarization are found.
- 13 Rotate QWP1 (still on the optical table) to one of the angles producing circular polarization (intensity minima) and do not change the angle again. Verify that the angle is correct with the polarization-checking tool. This waveplate angle is now set.
- 14 After verification of QWP1, place QWP0 back into the beam path as shown in Fig. 5d. Keeping the angle of QWP1 fixed, set QWP0 to one of the four fitted minimums that, based on fitting, produces circularly polarized light. This should position QWP0 such that the fast and slow axes are at 45° with respect to the linearly polarized laser beam.
- 15 Use the polarization checking tool to determine if the resulting linear polarization of the beam passing through both wave plates is at the same angle (vertical with respect to the optical table surface) as the input beam, or if the polarization has been rotated 90°. If the fast and slow axes of the QWPs are aligned, the result should be equivalent to a half waveplate, and the input polarization should be rotated by 90°. Alternatively, if the fast axis of QWP0 is aligned with the slow axis of QWP1, the two waveplates will cancel each other, and the input polarization will be unchanged at the output. The target is for the fast axis of QWP0 to be aligned with the fast axis of QWP1. When the two fast axes are aligned, the vertically polarized input beam will be rotated 90°. Thus, if the output polarization is the same as the input, rotate one of the waveplates by 90° to another intensity minimum. The QWP angles are now set for both QWP0 and QWP1, and do not change the angles.
- 16 Mount FAB-P0029 and FAB-OX0016 onto the vertical breadboard (FAB-OX0011).

Tower translation stage installation

Two long-travel linear translation stages (ASI and LS-50) are used to adjust the axial position of the sample stage and objective mounts (tower assembly). These two stages are electronically connected such that movement of one stage is synchronized with the other. Please note that this is a custom option and must be specified when ordering. Ideally, this results in smooth vertical movement of the tower assembly. However, it is necessary to install the stages and, before tightening the connecting bolts, allow the stages to travel through their range of motion. We recommend following this procedure:

- 1 Fully assemble the tower/stage stack following the CAD model (https://github.com/4Pi-SMS-consortium/CAD-files/blob/master/PDF/_ASY-StageAssemblies.pdf), as shown in Fig. 6.
- 2 Roughly position the tower/stage stack assembly on the optical table following the CAD model.
- 3 Slightly loosen the main connecting bolts, on both tower legs, highlighted in Fig. 6, taking care not to fully unscrew any (backing off 1/4 to 1/2 a turn from fully seated should be enough).
- 4 Translate the LS-50 stages through their full range of motion (up and down). **! CAUTION** Take care to avoid crashing the moving assembly into any components above or below.
- 5 Listen and feel for any binding or vibration while the stages translate. **▲ CRITICAL STEP** During steps 5–10, if binding or vibration is observed while the LS-50 stages are in motion, back off the connecting bolts and try slightly adjusting the relative positions of the supporting legs using the adjustment indicated in Fig. 6; then resume.
- 6 Very lightly seat all connecting bolts highlighted in Fig. 6.
- 7 Translate the LS-50 stages through their full range of motion again, taking care to avoid crashing.

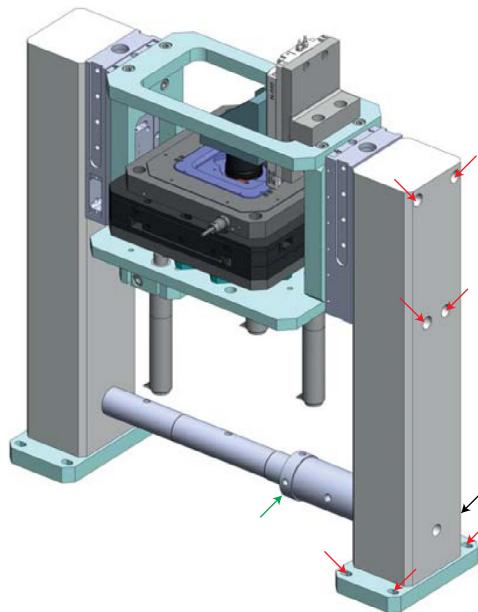


Fig. 6 | Microscope tower assembly and stage stack. M6 bolt locations loosened during LS-50 stage installation are indicated with red arrows for the right leg (the same positions for the left leg should also be loosened). An additional location, out of view, in the rear of the tower leg base is indicated with a black arrow. Fine adjustment between the tower legs is made with the telescoping base indicated with a green arrow.

- 8 Tighten the connecting bolts to medium tight.
- 9 Translate the LS-50 stages through their full range of motion again, taking care to avoid crashing.
- 10 Fully tighten all previously mentioned connecting bolts.
- 11 After all adjustments have been made, return the LS-50 stages to the axial position corresponding to the CAD model. This is easily accomplished by checking the offset of the stage platform relative to the stage body. The platform should be flush with the body (center of its range of motion) before starting the Procedure section.

NPBS installation

A nonpolarizing 50/50 beam splitter cube (NPBS, Fig. 1) is a central component of the microscope. The NPBS is glued to a custom plate (FAB-OX0006, Goniometer Cube Mount). We recommend using a UV-cured, low-shrinkage, optical adhesive (specifically, Norland Optical Adhesive 61). This adhesive is available directly from the manufacturer or from Thorlabs (part number NOA61). Although any appropriate UV source can be used to cure the adhesive, we recommend the curing system available from Thorlabs as part number CS2010. Lower-cost alternatives are available (e.g., McMaster-Carr, 1447T17). Care is needed to position the NPBS accurately so that the reflective surface sits along the horizontal symmetry plane of the system when fully mounted. We recommend the following procedure for assembly and gluing.

- 1 Attach rotation mount M-RS65 to the surface of a horizontal optical table or work surface.
- 2 Assemble connecting plate FAB-OX0005 with rotation mount M-RS65 and visually ensure that FAB-OX0005 is centered on the rotation platform.
- 3 Connect goniometer M-GON40-U to FAB-OX0005 and visually ensure that it is centered on the rotation platform.
- 4 Connect FAB-OX0006 (Goniometer Cube Mount) with goniometer M-GON40-U. Note that there are four registration tabs on the underside of FAB-OX0006, and these should be seated against the goniometer platform before fastening.
- 5 Level the goniometer platform.
- 6 Apply two to three drops of Norland Optical Adhesive 61 to the surface of FAB-OX0006 and place the NPBS (Edmund Optics, NT47-009) on top.
- 7 Remove any excess optical adhesive.
- 8 Apply light pressure to seat the NPBS cube against the registration tabs on FAB-OX0006 as indicated in Fig. 7.

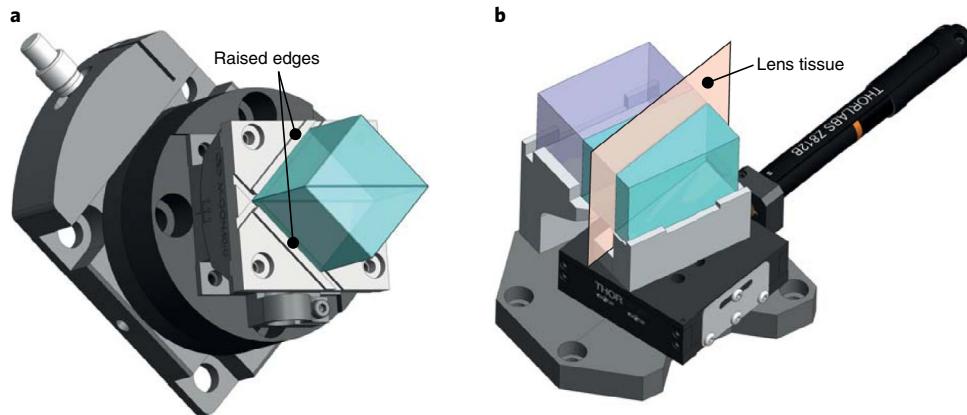


Fig. 7 | NPBS and wedge assembly. **a**, Two registration tabs (raised edges, indicated with arrows) are provided to seat the NBPS cube against before adhering to its mount. **b**, Before gluing the optical flat and wedge pair, it is helpful to place a lens tissue between the wedge pair to ensure that they are parallel, are not touching and have minimal separation.

- 9 Irradiate the optical adhesive with the UV curing system (at 90% intensity; if Thorlabs CS2010 is used, 2–3 min should be enough). **! CAUTION** Parts attached with only partially cured UV glue can spontaneously detach and fall when mounted vertically in the microscope. **! CAUTION** The UV-cured optical adhesive recommended here cannot be removed or easily dissolved with a solvent. Be certain about optic placement and orientation before you cure the adhesive.

PBS cube installation

Installation of the PBS cube (PBS0, Fig. 1) uses a UV-cured, low-shrinkage optical adhesive and UV curing light source as outlined above. We recommend the following procedure for assembly:

- 1 Mount the prism mount (New Focus, part number 9481) horizontally on an optical table and ensure that its platform surface is level.
- 2 Remove the retaining arm. It is not needed, as the optic will be glued in place.
- 3 Apply two to three drops of Norland Optical Adhesive 61 to the surface of the prism mount and place PBS0 (Edmund Optics, NT49-002) on top.
- 4 Using the white placement markers on the platform surface, center PBS0 on the platform.
- 5 Irradiate the optical adhesive with the UV curing system (if Thorlabs CS2010 is used, 2–3 min should be enough). **! CAUTION** The UV-cured optical adhesive recommended here cannot be removed or easily dissolved with a solvent. Be certain about optic placement and orientation before you cure the adhesive.

Quartz and BK7 wedge/flat assembly installation

The microscope uses a flat and prism pair in both the upper and lower interference cavity paths (Fig. 1a). The BK7 flat with quartz wedge pair may be installed in either the upper or lower path. Here, we presume that the BK7 flat and quartz wedge pair are installed in the upper path (W0, Fig. 1a and Fig. 7b) and that the quartz flat and BK7 wedge pair are installed in the lower path (W1, Fig. 1). Here, we present installation instructions for the BK7 flat and quartz wedge pair (W0) in the upper path. The same procedure applies to W1 in the lower path. Installation should be carried out on a horizontal optical table or work surface, not on the vertical breadboard of the microscope. It is important to note that, when the custom quartz and BK7 optics arrive, their respective shipping containers will be labeled, but the individual optics will not. Use a pencil and write the designation on the unpolished surface of each part to avoid confusion later.

- 1 Install motorized translation stage MT1/M-Z8 onto FAB-OX0004 (Wedge-Upper Baseplate) using alignment pins supplied with MT1/M-Z8.
- 2 Ensure that translation stage MT1/M-Z8 is visually in its center position and that the corresponding servo motor (Z812B, as a part of MT1/M-Z8) is installed.
- 3 Attach FAB-P0048 (Wedge-Only Mount Upper) to the platform of MT1/M-Z8.
- 4 Attach FAB-P0051 (Wedge Support Large) to FAB-OX0004 (Wedge-Upper Baseplate).
- 5 Clean the mounting surfaces of FAB-P0048 and FAB-P0051 with ethanol and allow adequate time to dry.

- 6 Apply four or five drops of optical adhesive, as detailed above, to the mounting surface of FAB-P0051 and place the bonded BK7 flat and quartz wedge onto the mounting surface.
- 7 Using nylon-tipped set screws, apply very light lateral pressure to FAB-P0051 and ensure that it is firmly seated against the three registration tabs.
- 8 Use the UV cure gun for 2–3 min at 90% intensity to cure the optical adhesive. **!CAUTION** The UV-cured optical adhesive recommended here cannot be removed or easily dissolved with a solvent. Be certain about optic placement and orientation before you cure the adhesive.
- 9 Physically confirm that the UV adhesive has formed a strong bond by attempting to remove the optic from the mount. If the optic detaches, clean the mounting surface and optic and return to step 7 above.
- 10 Apply two small drops of UV optical adhesive to the mounting surface of FAB-P0048 and place the remaining wedge on top.
- 11 Place a single piece of lens tissue between the wedges, as shown in Fig. 7b, and ensure that the unglued wedge is seated against the angled surface of the already-installed wedge.
- 12 Use the UV cure gun to fix the unattached wedge.
- 13 Remove the lens tissue and physically confirm that a strong bond has been formed.

Cavity dichroic beam splitter and emission filter installation

A pair of custom-cut dichroic beam splitters, DI0 and DI1 (Chroma, ZT405/488/561/640rpcv2-UF1), and a pair of matched emission filters (Chroma ZET405/488/561/640mv2) are glued into respective custom filter cubes (FAB-P0032) with one sitting above the upper objective lens (OBJ0) and the other below the lower objective lens (OBJ1). These optics should be glued in place following the CAD model (https://github.com/4Pi-SMS-consortium/CAD-files/blob/master/4Pi-Oxford-Packed/Workspaces/Workspace/4Pi-Oxford/_TOP_4Pi-Oxford.iam). Although a UV optical adhesive may be used, as in previous sections, we recommend using a two-component silicone-based adhesive, TwinSil (Picodent, 1300 1000). This has the advantage of being strong enough to hold the optics in place while also affording the possibility of later removal if the microscope is modified for other color channels. **▲CRITICAL** For both the upper and lower filter/dichroic set, proper orientation of both optics is important. Once installed, the filter cubes are not easily removed for correction.

Image-combining prism installation

The final reflective element before fluorescence emission reaches the imaging camera is a knife-edge right angle prism (Thorlabs, MRAK25-P01). This prism is glued onto its custom mount, FAB-P0039. The prism is bonded, using the same optical adhesive and UV cure gun as detailed above, according to the following procedure:

- 1 Connect FAB-P0039 (Combining Mirror Mt) to a horizontal optical table or other flat, level workspace.
- 2 Clean the mounting surface of FAB-P0039 with ethanol and allow adequate time to dry.
- 3 Apply two small drops of UV optical adhesive to the mounting surface of FAB-P0039 and place the prism top.
- 4 Ensure that the prism is firmly seated against the two positioning registration tabs.
- 5 Irradiate the optical adhesive with the UV curing system (if Thorlabs CS2010 used, 2–3 min should be enough). **!CAUTION** The UV-cured optical adhesive recommended here cannot be removed or easily dissolved with a solvent. Be certain about optic placement and orientation before you cure the adhesive.
- 6 Physically confirm that a strong bond has been achieved before mounting on the vertical breadboard.

DM installation

Two DMs are mounted in five-axis mounts (Newport, LP-2A) as shown in Fig. 8a,b. Each mount is subsequently mounted vertically on FAB-OX0011 (Plate 1) with installation and location following directly from the CAD model. However, several important details are easily overlooked and, thus, highlighted here:

- The DMs ship with four ribbon cables attached. The cable plug-ends are too large for an LP-2A retaining ring to fit directly onto the cables (see Fig. 8a for the desired arrangement). Thus, it is necessary to cut one LP-2A retaining ring. This can be accomplished with a razor blade or very thin saw blade. Once the ring is cut, slip it over the ribbon cables and use a small amount of two-component epoxy or another strong adhesive to repair the ring. Alternatively, it is possible to remove the DM back plate and disconnect the ribbon cables. **!CAUTION** We strongly recommend

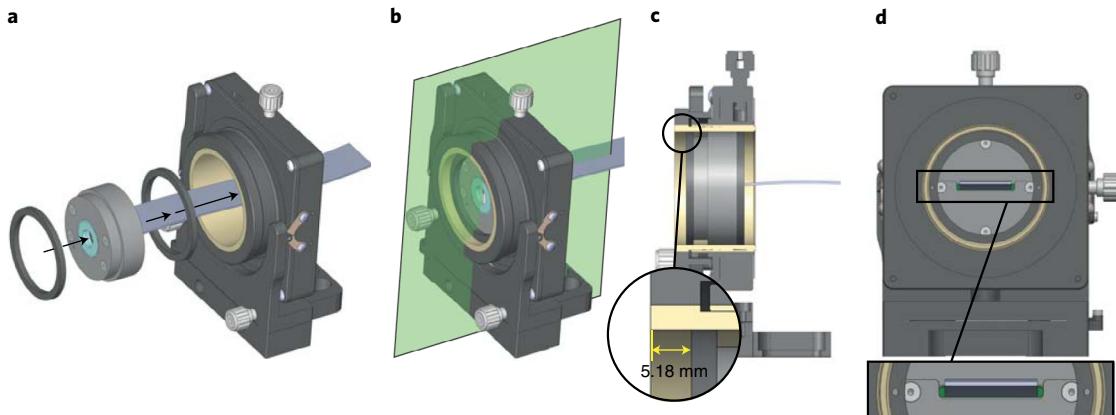


Fig. 8 | DM installation. **a**, Overview of inserting a DM into a mount (LP-2A-XYZ, Newport). The front mounting bracket is attached to the opposite side as used here and must be switched. **b**, The DM installed in the LP-2A mount with the plane for a cross-sectional view (green) shown in **c**. Ensure that the front retaining ring is installed at the depth shown in **c** and that the brass housing is flush with the mount body. This ensures that the DM is seated at the correct starting position. **d**, The orientation of the DM can be observed by inspecting the cable aperture in the back plate.

against removing the back plate, because of an extremely high risk of mirror damage from static discharge. Even one damaged actuator can have a detrimental effect on the final system PSF. Depending on the purchasing timeline, the LP-2A mount may be purchased early enough that a retaining ring can be sent to the vendor for installation.

- As presented in Fig. 8c, each DM should be mounted at a specific depth inside the LP-2A mount to ensure a good axial starting position for system setup. The axial position of the DM will be tuned once inside the system. Please also note the initial position of the LP-2A mount axial adjuster following from the CAD model (https://github.com/4Pi-SMS-consortium/CAD-files/blob/master/4Pi-Oxford-Packed/Workspaces/Workspace/4Pi-Oxford/_TOP_4Pi-Oxford.iam).
 - Each DM has a protective glass window sitting in front of a reflective deformable surface. These windows are seated at a small angle, making the rotational orientation of the DM important. We recommend reviewing the window tilt position relative to the DM location within the CAD model. The orientation of the DM back plate may be a good point for reference as presented in Fig. 8d.
- ▲ CRITICAL** DMs may be ordered with a custom split back plate that allows the attached ribbon cables to exit the mirror body from a central rear aperture. Each DM must be ordered with this option; otherwise, it is not possible to seat them in the five-axis LP-2A mount. **▲ CRITICAL** We recommend using an interferometer to check that each DM is working as expected before use. This can be accomplished by mounting the DM on an optical table and setting up a small interferometer or using a slot-in interferometer²⁴.

DM calibration

DM calibration involves measuring changes in the mirror's reflective membrane relative to a desired shape. These measurements are used to create a set of orthogonal modes (shapes) that can be used later to control the reflective membrane's shape for aberration correction. This process requires the use of an interferometer to measure changes to the reflective membrane relative to a flat reference mirror. Full details on calibration and construction of a 'slot-in' interferometer can be found at ref. ²⁴. We recommend carefully reading the calibration instructions, ordering parts for a slot-in interferometer and making sure that all required software is installed and available before starting Steps 220–233.

Optomechanical assembly

System setup follows directly from the CAD model (https://github.com/4Pi-SMS-consortium/CAD-files/blob/master/4Pi-Oxford-Packed/Workspaces/Workspace/4Pi-Oxford/_TOP_4Pi-Oxford.iam). Although several free CAD viewers are available (in this case, Autodesk Inventor View is the recommended choice), these are typically limited to basic part or assembly viewing and lack any functionality to hide or show parts or view part and assembly cross sections (useful for checking lens orientation and mounting depth). Here, we assume access to a working version of Autodesk Inventor for viewing how individual components are assembled. **▲ CRITICAL** As custom and commercial parts arrive, assemble the microscope following the CAD model. Install all stages,

lenses, filters, light sources, beam splitters, alignment pins and cameras. Pay close attention to details such as lens mounting depth in holders that do not have a defined mechanical stop and lens and filter orientation. **▲CRITICAL** Carefully select the installation location on the optical table for the vertical breadboard and tower assembly to ensure adequate space on all sides of the system. Positioning the tower assembly near the optical table edge is advised for easy sample loading/removal. **▲CRITICAL** Objective lens OBJ1 (lower) is connected to an XY piezo stage (PI, P-612) for lateral positioning. On several existing systems, this stage has had significant vibration in closed loop mode when the large mass of the objective is installed. This is fixed by changing physical settings (potentiometer adjustments) inside the controller. We recommend working with PI, ahead of delivery, to tune the stage for high stability (and slow step response) when horizontally mounted with an approximately 200-g rigid mass attached. Alternatively, the necessary parameters can be adjusted once the stage is installed.

Sample stage leveling

The sample stage must be levelled. The sample XY and Z stages are supported from below with three motorized actuators (PI, M227.10). These three actuators control the sample coarse Z-position and the sample level. Use a bubble level (e.g., Thorlabs part number LVL01) and adjust the actuators until the sample stage is level. In addition, adjust the sample stage Z-position to roughly match the position in the CAD model (https://github.com/4Pi-SMS-consortium/CAD-files/blob/master/4Pi-Oxford-Packed/Workspaces/Workspace/4Pi-Oxford/_TOP_4Pi-Oxford.iam) using the M227 DC mike motors.

Primary imaging camera orientation

The primary imaging camera, Hamamatsu ORCA-Flash4.0 V3 Digital CMOS (ORCA FLASH C11440-22CU, Hamamatsu Photonics), on the microscope uses a rolling shutter to achieve fast frame rates. In this mode, the central rows of the camera sensor are exposed first, and exposure progresses from the center rows to the edge rows in two directions. Thus, when imaging at high frame rates, it is important to have the central exposure line of the camera positioned through the center of all four interference images to ensure simultaneous exposure. In practice, this means the orientation of the camera should follow from the CAD model (https://github.com/4Pi-SMS-consortium/CAD-files/blob/master/4Pi-Oxford-Packed/Workspaces/Workspace/4Pi-Oxford/_TOP_4Pi-Oxford.iam) with the camera cooler connections in the ‘top’ (or ‘bottom’) position. **▲CRITICAL** The primary imaging camera (Hamamatsu, Orca Flash) used in this system has two cooling options: an internal fan or liquid cooling using an external source. Using the internal fan will cause vibrations, resulting in an effective loss of interference. Thus, an external liquid cooler is required. Many suitable liquid coolers are available (e.g., EXOS-2, Koolance).

Device drivers and software

Before starting the alignment protocol, all computer-controlled devices should be connected to the acquisition computer, manufacturer-supplied drivers and control software should be installed and their operation should be verified. Manufacturer-supplied software to install includes the following:

- Hamamatsu, DCAMAPI and HCImage Live for acquisition camera control
- IDS Imaging, uEye camera drivers and camera manager for alignment camera control (if following recommendations above)
- ASI, ASIConsole for LS-50 linear translation stage control
- Thorlabs, Kinesis for quartz and BK7 wedge motor control, or IonStudio if using RoboClaw controllers
- PI, PIMikroMove for piezo, sample, objective Z and DC-Mike control

Microscope control software

Three software packages from National Instruments (NI) Corporation should be installed on the data-acquisition computer for the LabVIEW-based microscope control software. These are:

- NI LabVIEW 2016, 64-bit
- NI Device Drivers (DAQmx and virtual instrument software architecture (VISA) are required)
- NI Vision Development Module

Software control of the microscope is carried out using a custom LabVIEW-based control program. The latest version of the software is hosted at <https://github.com/Gurdon-Super-Res-Lab/Microscope-Control> and is currently used across multiple institutions. Specific instructions for

installation of the LabVIEW-based control software can be found on the associated YouTube channel (www.youtube.com/channel/UC7R7VayC6sGlrphbH7pn-w/).

Data analysis software

Several software packages should be installed on the data analysis computer for the MATLAB-based analysis program. These are:

- MATLAB 2016a or later, 64-bit
- The latest Nvidia graphics driver
- CUDA Toolkit 4.2.9 or later, 64-bit
- DIPImage, 64-bit

Custom data analysis software is written in MATLAB and hosted at <https://github.com/4Pi-SMS-consortium/4Pi-SMS-analysis>.

Procedure

Software installation and setup ● Timing 5–8 d

- 1 Connect all computer-controlled devices (e.g., stages, lasers and cameras) to the acquisition computer and install all manufacturer-supplied drivers and control software listed in ‘Equipment setup’. Verify that all devices are working correctly.
- 2 Install custom microscope control software, as detailed in ‘Equipment setup’.

Optomechanical assembly ● Timing 5–8 d

- 3 If not already done, glue all optical components to their respective mounts as described in ‘Equipment setup’ and install the assemblies on the vertical breadboard.
- 4 If not already done, ensure that all component assembly described in ‘Equipment setup’ has been completed.
- 5 If not already done, install all stages, lenses, filters, light sources, beam splitters, alignment pins, cameras and mechanical components following directly from the CAD model found at <https://github.com/4Pi-SMS-consortium/CAD-files> and details found in ‘Equipment setup’. Pay close attention to details such as lens mounting depth in holders that do not have a defined mechanical stop and lens and filter orientation. Couple all excitation lasers into a single mode fiber.

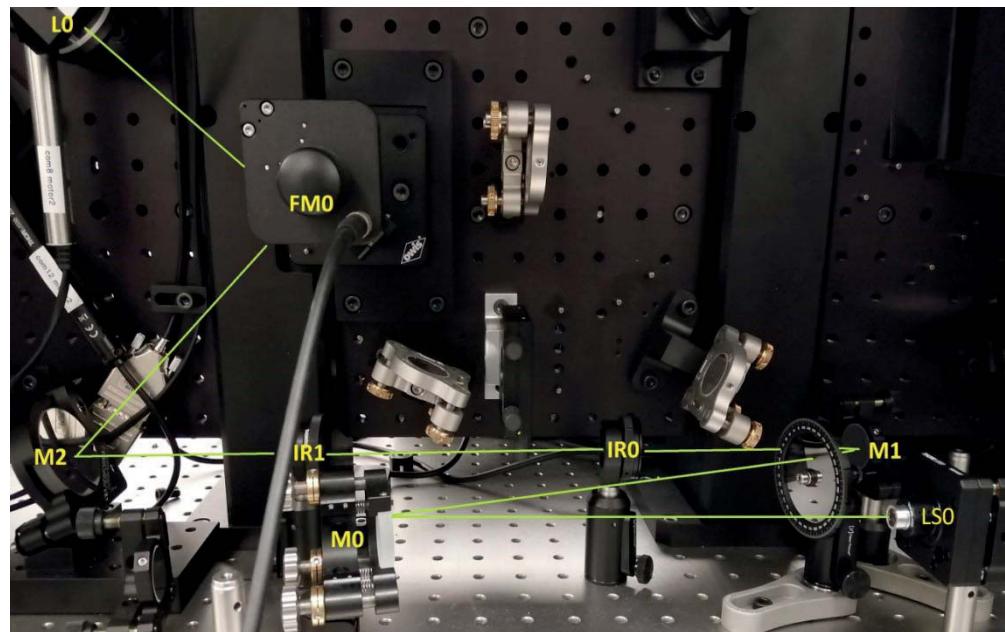


Fig. 9 | Alignment laser setup. An alignment laser (LSO) is launched at two fold mirrors (M0 and M1) and directed through two irises, IRO and IR1. It reflects from a 45° mirror (M2) to a flip mirror (FMO), where it reflects and enters the interference cavity for alignment.

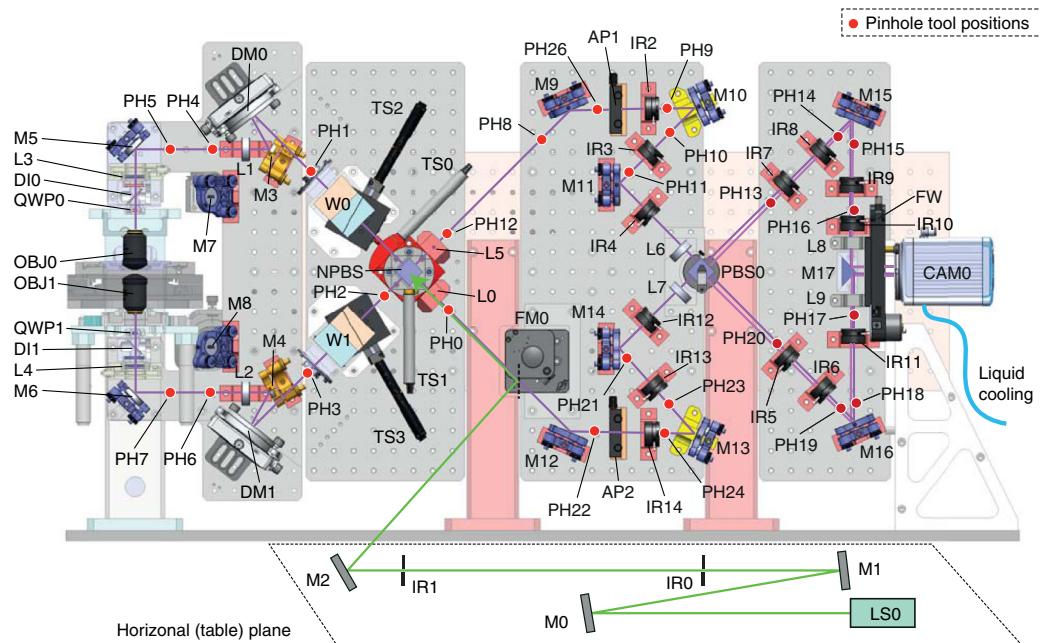


Fig. 10 | Labeled diagram of the 4Pi-SMS emission path. AP1 and AP2, apertures; CAM0, primary imaging sCMOS camera; D10 and D11, dichroic beam splitters; DM0 and DM1, deformable mirrors; FW, filter wheel; IRO-14, iris diaphragms; L0-9, lenses; LS0, alignment laser; M0-17, mirrors; OBJ0 and OBJ1, objective lenses; PHO-24, pinhole tool positions (highlighted with red dots) with dowels; QWPO and QWP1, quarter waveplates; TSO-3, linear actuators; W0 and W1, wedge/flat assemblies.

Alignment laser setup ● Timing 4-8 h

- 6 Select an appropriate alignment laser following the guidelines described in ‘Experimental design’.
 - 7 Install a fold mirror at 45° (M2, Figs. 9 and 10), with respect to the optical table surface, at the location that light reflected from FM0 will intersect the optical table following from Fig. 9.
 - 8 Arrange two iris diaphragms along a row of holes on the horizontal optical table, roughly matching the layout in Fig. 9. The CAD model (https://github.com/4Pi-SMS-consortium/CAD-files/blob/master/4Pi-Oxford-Packed/Workspaces/Workspace/4Pi-Oxford/_TOP_4Pi-Oxford.iam) shows that the beam path on the vertical breadboard sits directly over a row of holes on the optical table when viewed from above. Install the irises using this row of holes after M2 following from Fig. 9. Make sure that both irises have the same height.
 - 9 Arrange two mirrors roughly matching the layout in Fig. 9, with heights matching the irises, IR0 and IR1.
 - 10 Launch the alignment laser at the fold mirrors such that it roughly propagates through both IR0 and IR1, reflects from M2 and continues onto FM0.
 - 11 Use the iterative alignment procedure in Box 1 (General Beam Alignment Procedure) to set mirrors M0 and M1 so that the alignment laser passes the centers of IR0 and IR1.

Interference cavity alignment ● Timing 2-4 weeks

▲CRITICAL This section refers to component and location designations (e.g., M1, M2, PH0, PH1 and so on) as shown in Figs. 1 and 10.

- 12 Ensure that the alignment laser propagates approximately from the center of M2 to the center of FM0. Read the relevant portions of ‘Overview of the procedure’, if you have not already done so.
 - 13 (Optional) If you are using the optional 3D-printed shutter mounts, skip Steps 13 and 14 and proceed to Step 15. Otherwise, remove the shutter SH0 and shutter mount (FAB-CAM005) from the wedge and flat assembly at position W0, leaving the wedge/flat assembly in place. Mirror M3 and its mount may need to be temporarily removed to access the connecting bolts.
 - 14 (Optional) Remove the shutter SH1 and shutter mount (FAB-CAM005) from the wedge/flat assembly at position W1, leaving the wedge/flat assembly in place. Mirror M4 and its mount may need to be temporarily removed to access the connecting bolts.
 - 15 Remove lens L0 and insert pinhole alignment tools into positions PH0 and PH1.

- 16 Use mirrors M2 and FM0 to align the alignment laser with the centers of PH0 and PH1 via the iterative alignment procedure in Box 1.
▲ **Critical Step** Flip mirror FM0 has a limited tip and tilt range. Ensure that the alignment laser is close to the centers of PH0 and PH1 before starting this step.
- 17 Once aligned with PH0 and PH1 centers, remove the pinhole tool from position PH0. The alignment laser should now pass through W0.
- 18 Insert lens L0 and check that the focused beam is centered on PH1. If so, remove L0. If the beam is not centered, check again that the alignment beam passes the center of PH0 and PH1.
- 19 Remove both pinhole tools from locations PH0 and PH1. The alignment laser should now reach DM0.
- 20 Install pinhole alignment tools at positions PH2 and PH3.
- 21 Using servo motors TS0 and TS1, adjust the tip and/or tilt of the NPBS's reflective surface until the alignment laser passes through the centers of PH2 and PH3. TS0 and TS1 control the tip and tilt of the NPBS like that of a tip-tilt mirror mount. Because the alignment laser beam leaves FM0 on the correct path, from Step 16, the beam should be able to pass the center positions of PH2 and PH3 by adjusting the tip and/or tilt of the NPBS only. If this cannot be achieved, the position of the NPBS is not correct, and the position of the reflective surface of the NPBS relative to the symmetry plane of the system must be adjusted. To correct this, adjust the lateral position of the mount (Newport, M-GON40-U) using the play in the fastening holes. The adjustment will be very small. Once the transmitted beam passes through the center of PH1 and the reflected beam is incident on PH2 and PH3 in their respective centers, the NPBS is aligned well.
- 22 Remove both pinhole tools from locations PH2 and PH3. The alignment laser should now reflect from the NPBS and reach DM1, while the transmitted portion of the alignment laser should reach DM0.
- 23 Install shutters SH0 and SH1 and open both.
- 24 Remove lenses L1 and L3 and objective lens OBJ0.
- 25 Using a paper card or target, adjust mirrors M3 and M5 until the alignment laser beam roughly passes through the center of the mount for OBJ0.
- 26 Mount the alignment camera, as described in 'Experimental design', in the mount for OBJ0 such that the alignment laser beam reflected from M5 is incident on the sensor surface. This is easily done using an SM1-to-RMS (Royal Microscopical Society) thread adaptor (e.g., Thorlabs part number SM1BC or SM1A4).
- 27 With the alignment camera connected to a computer, open the appropriate software (e.g., ThorCam from Thorlabs or uEye Camera Manager from IDS) to see the camera image in real time (live preview mode). Adjust the camera exposure time or the alignment laser intensity so that the camera sensor is not saturated.
- 28 Using the DM control software¹⁹, poke (move) the center actuators of the DM or apply a radially symmetric pattern to the DM as shown in Fig. 11a. Apply enough stroke (~1.25 μm) to the active actuators so that they can be seen on the camera.
- 29 Using the lateral XY adjustment screws on the LP-2A mount, move the DM laterally until the center actuators are (visually) coincident with the center of the alignment laser as seen in Fig. 11b. Fine lateral adjustment of the DM is not necessary at this point and will be revisited in Steps 222–233 with fluorescence bead images.
- 30 Repeat Steps 24–29 for DM1 (lower). That is, remove lenses L2 and L4 and objective OB1, mount the alignment camera in the mount for objective OB1 such that the beam reflected by M6 is incident on the camera sensor and follow the same procedure for centering DM1.
- 31 Install pinhole alignment tools at positions PH4 and PH5. Lens L1 should still be uninstalled.
- 32 Use the beam alignment procedure in Box 1, in combination with mirrors DM0 and M3, and align the alignment laser beam with the centers of pinholes at PH4 and PH5.
- 33 Insert lens L1 and confirm that the focused beam is centered on PH4 and PH5. If the beam is not centered, adjust the position of the lens L1 in its mount (LMR1) by loosening the retaining ring and adjusting the lens position until the focused beam is centered on PH4 and PH5. There is ~400 μm of play between the lens and the mount.
- 34 Remove lens L1.
- 35 Install pinhole alignment tools at positions PH6 and PH7. Lens L2 should still be uninstalled.
- 36 Use the beam alignment procedure in Box 1, in combination with mirrors DM1 and M4, to align the alignment laser beam with the centers of pinholes at PH6 and PH7.
- 37 Repeat Step 33 for lens L2 and pinholes PH6 and PH7.
- 38 Remove lens L2. Lenses L3 and L4 and both objective lenses should remain uninstalled.

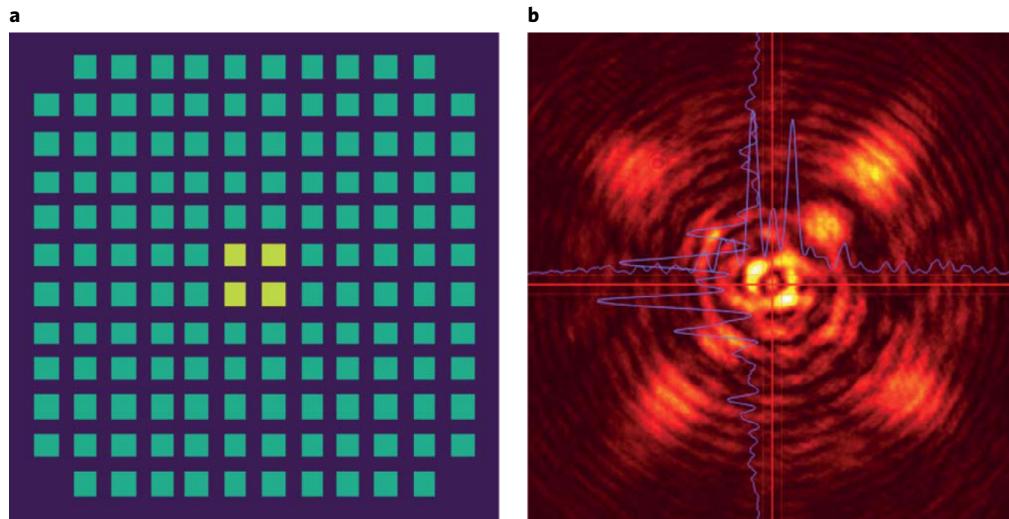


Fig. 11 | DM control pattern used for centering. **a**, DM control pattern: a 1.25- μm stroke is applied to the center 2×2 actuators (yellow). **b**, An example beam profile recorded on the alignment camera when the alignment laser hits the DM center.

- 39 Install the alignment camera (see ‘Experimental design’) into OBJ0’s mount such that the beam reflected from M5 is incident on the camera sensor.
- 40 Move the translation stage holding OBJ0’s mount (N-565) to its maximum possible vertical position.
! CAUTION Use caution when determining the maximum vertical position of the objective mount, as it may crash into components above.
- 41 Mark the incident position of the alignment laser beam on the alignment camera’s sensor.
- 42 Move translation stage N-565 to its lowest possible vertical position.
! CAUTION Use caution when determining the minimum vertical position, as the alignment camera may crash into components below.
- 43 Use the tip and/or tilt adjustments on mirror M5 to visually bring the center of the alignment laser to the position marked in Step 41.
- 44 Repeat Steps 40–43 until no lateral beam displacement is observed when translating the alignment camera from the maximum to the minimum vertical position. Many iterations will be required. Once complete, the alignment laser beam should leave mirror M5 perpendicular to the optical table surface.
- 45 Remove the alignment camera from OBJ0’s mount. If using the compact camera mount described in ‘Experimental design’, you can remove the camera from the mount and flip the printed circuit board (PCB) such that the sensor faces away from the SM1 threads. Do this and reinstall the camera onto OBJ0’s mount. The camera sensor will now be facing down such that the reflected beam from mirror M6 is incident on its surface. If using a different camera and/or camera mount, figure out how to mount the camera in the same way.
- 46 With the alignment camera facing mirror M6, repeat Steps 40–44 using mirror M6 until no lateral displacement of the alignment laser is observed when translating the alignment camera between the maximum and minimum axial positions. Once complete, the alignment laser beam should leave mirror M6 perpendicular to the optical table surface.
- 47 With the alignment camera still facing mirror M6, loosen the four M6 bolts on each tower support foot (FAB-P0001, Vertical Support Base).
- 48 The loosened M6 bolts in Step 47 connect to the optical table through short slots in FAB-P0001 that allow the entire tower assembly to be translated in one dimension, along the slot’s long axis. Adjust the tower position along the M6 slots until the alignment laser is centered on the alignment camera sensor in the corresponding dimension.
- 49 Tighten the M6 bolts that were loosened in Step 47. The tower assembly should now be positioned such that the alignment laser beams reflecting from mirrors M5 and M6 pass the center of OBJ0’s mount in the dimension adjusted during Step 48.
- 50 The kinematic mount for mirror M6 has three adjustment screws that allow the mirror surface to be translated in one dimension, the dimension orthogonal to the attachment screw slots used in

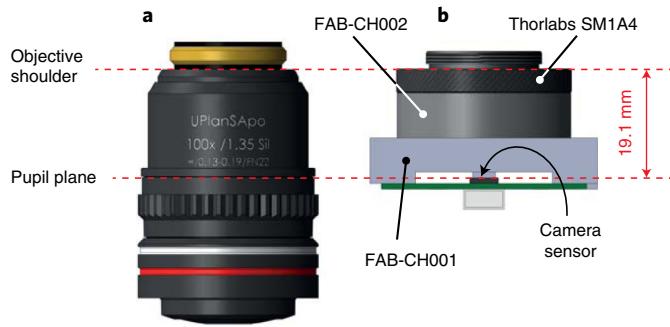


Fig. 12 | Objective pupil plane location and alignment camera setup. **a**, The objective pupil position is 19.1 mm inside the objective measured from the objective shoulder. **b**, Alignment camera mounted on a compact holder with RMS thread adaptor such that the camera sensor sits at the position of the objective pupil plane relative to the RMS thread shoulder.

- Steps 47–49. Adjust these three screws equally until the alignment laser beam is centered on the alignment camera sensor in the corresponding dimension. This adjustment will probably cause the alignment laser beam reflected from M6 to no longer be perpendicular to the optical table surface. Repeat Steps 40–44 using mirror M6 until the beam is again perpendicular to the optical table surface. If necessary, adjust the three screws on M6 to center the alignment laser beam, in one dimension, on the alignment camera sensor again.
- 51 The alignment laser beam reflecting from M6 to the alignment camera should now be centered on the sensor. Install lens L4 and adjust the lateral position of the lens until the focused beam is centered on the alignment camera sensor.
 - 52 Flip the alignment camera in the compact mount (following from Step 45), such that the camera sensor faces the SM1 thread, and reseat that alignment camera in OBJ0’s mounting bracket.
 - 53 Repeat Step 50 with mirror M5.
 - 54 Install lens L3 and repeat Step 51 with lens L3.
 - 55 Remove the alignment camera from OBJ0’s mounting position.
 - 56 The back-pupil plane of the 100× Olympus objectives used in the microscope sit 19.1 mm inside the objective from the shoulder as shown in Fig. 12a. Position the alignment camera sensor 19.1 mm from the OBJ0 objective mount shoulder. This is accomplished using the compact camera mount and tube connector as seen in Fig. 12b. Mount the alignment camera as shown in Fig. 12b or via an alternative solution if a different camera is used.
 - 57 Install the alignment camera, as mounted in Step 56, into OBJ1’s mount. The alignment camera should now be mounted with its sensor sitting where objective OBJ1’s back-pupil plane will reside.
 - 58 Set both axes of the XY piezo stage holding OBJ1’s mount to their center positions.
 - 59 Two spring-loaded adjustable pushers hold OBJ1’s XY piezo stage in place. The two spring-loaded pushers each include an adjustment screw. Adjust the position of OBJ1’s XY piezo stage with the adjustment screws until the center of the alignment camera sensor is visually centered with the alignment beam. OBJ1’s mount should now be approximately centered. The position will be fine-tuned later using a fluorescent bead sample.
 - 60 Install lenses L2 and L4.
 - 61 With lenses L2 and L4 installed, the surface of DM1 is imaged onto the alignment camera. When the DM’s surface is slightly displaced from in focus, a grid of rectangles will be visible as seen in Fig. 13a. Adjust the axial position of the DM1 using the LP-2A mount until the grid is no longer visible and connection wires are in focus as seen in Fig. 13b.
 - 62 The DM1’s axial position should require minimal adjustment. However, if the DM is translated over a larger distance (>1 mm), some displacement in the alignment laser beam will occur. For this reason, remove L2 and confirm that the alignment laser beam is still reasonably collinear with pinholes at locations PH6 and PH7 after bringing the DM into focus.
 - 63 Remove the alignment camera from OBJ1’s mount and, following from Step 57, mount the camera into OBJ0’s mount so that the sensor is positioned at the objective’s back-pupil plane position.
 - 64 Translate OBJ0’s Z stage (N-565) until the platform position matches the position in the CAD model. The alignment camera’s sensor should now be sitting at the location of OBJ0’s back-pupil plane.
 - 65 Install lenses L1 and L3.

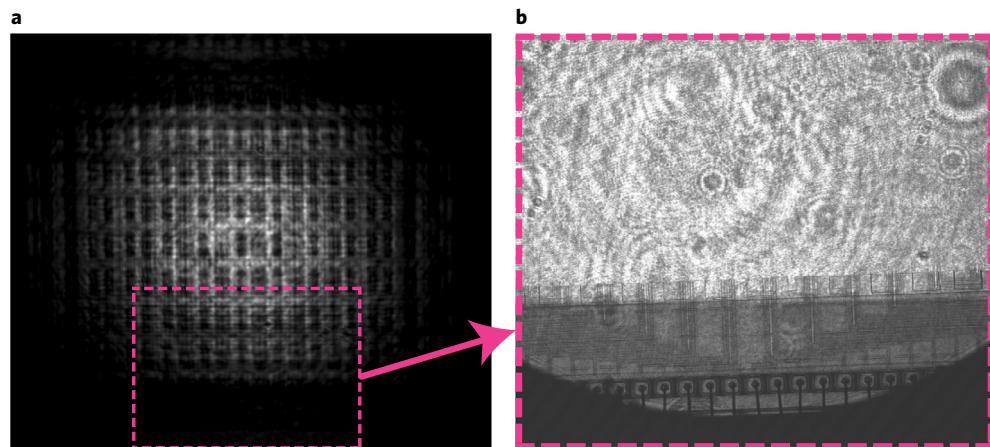


Fig. 13 | DM conjugation. **a**, Image of the full DM surface when the camera sensor and DM membrane are not conjugated. **b**, Zoom-in image of the DM surface when the camera sensor and the DM surface are conjugated (the corresponding region is shown in the purple box in **a**). When conjugated correctly, the pattern from the DM surface (bright area) can no longer be seen while the fine connection wires and electrodes at the edge of the reflective membrane are in focus (lower portion of **b**).

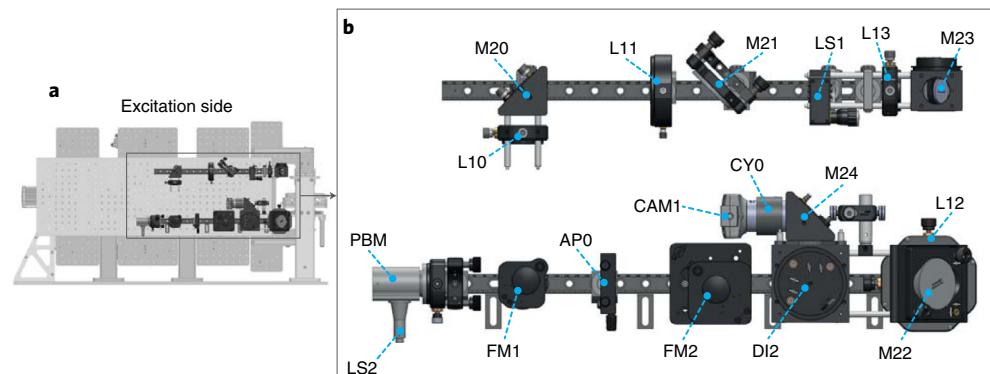


Fig. 14 | Excitation path diagram with labels used throughout the alignment procedure. AP0, adjustable aperture; CAM1, USB CMOS camera (DCC1545M, Thorlabs); CY0, cylindrical lens; DI2, short-pass dichroic beam splitter (DMSP805L, Thorlabs); FM1 and FM2, flip mirrors; L10–13, achromatic lenses; LS1, 940-nm laser (LP940-SF30, Thorlabs); LS2, fiber from multi-color laser module including both a 642-nm laser and an activation 405-nm laser; M20–24, silver mirrors; PBM, parabolic mirror.

- 66 Following from Step 61, adjust the axial position of DM0 until its surface is brought into focus on the alignment camera.
- 67 Confirm that after DM0 axial adjustment, the alignment beam is still collinear with pinholes at positions PH4 and PH5.
- 68 Remove the alignment camera.

Excitation path alignment ● Timing 1–2 weeks

▲ CRITICAL The procedure described here presumes that all lasers have been coupled into a single-mode fiber, LS2 (Fig. 14), as the starting point. We also presume that all optics, optomechanics and other elements of the excitation path have been assembled and mounted on the system following the CAD model.

▲ CRITICAL This section refers to component and location designations (e.g., M1, M2, LS1, LS2 and so on) as shown in Fig. 14.

- 69 Read the ‘Excitation path alignment’ subsection of ‘Overview of the procedure’ and the ‘Laser safety’ subsection of ‘Experimental design’, if you have not already done so.
- 70 Use a ruler or callipers and open AP0 to ~4.5 mm square.

- 71 Switch on a low-power light source and adjust the power such that light exiting the fiber (LS2) is within safety regulations for open beam work. This source will be used as the alignment beam for the excitation path.
- 72 If not already done, connect the single-mode fiber carrying the excitation light to the reflective collimator parabolic mirror (PBM).
- 73 Confirm that flip mirrors FM1 and FM2 are positioning so that the excitation light exiting the collimator proceeds along a straight path to mirror M22.
- 74 Adjust the tip and/or tilt and translation on the laser launcher mount, LS2, such that the excitation beam hits the center of aperture AP0 and the center of mirror M22.
- 75 Remove L12 and its XY translation mount from the rails after M22. This allows the excitation beam to reflect from mirror M22 and onto M8 (located on the ‘emission’ side of the vertical breadboard; Fig. 2).
- 76 Remove both objectives, if installed, and place a piece of paper or install a ground glass alignment disk (e.g., Thorlabs VRC2RMS) on top of the lower objective (OBJ1) mount.
- 77 Adjust M22 to bring the excitation beam to the center of OBJ1’s mount.
- 78 Remove the paper or alignment disk and install it on the upper objective (OBJ0) mount.
- 79 Adjust M8 to bring the excitation laser to the center of the objective mount.
- 80 Repeat Steps 76–79, following the beam alignment procedure in Box 1, until the excitation beam passes through the center of both the upper (OBJ0) and lower (OBJ1) objective mounts.
- 81 Install objective OBJ1 (lower).
- 82 Again, use a piece of paper or install an alignment target on the upper objective mount to check if the focused beam passes the objective mount center. If not, adjust M22 and M8 to center it.
- 83 Remove OBJ1 to check that the excitation beam is centered within the lower objective (OBJ1) mount aperture.
- 84 Install OBJ1 and mark the position the excitation beam hits the target on the upper objective (OBJ0) mount.
- 85 Center L12 in its XY translation mount using the white crosshairs on the mount.
- 86 Install L2 and its XY translation mount onto the 60-mm cage rails adjacent to M22 and following the CAD model for its axial location.
- 87 Adjust the lateral position of L12 until the excitation beam exiting OBJ1 is incident on the marked position in Step 84.
- 88 Remove the target or card from OBJ0’s mount and allow the beam to reach mirror M7.
- 89 Adjust M7 so that the beam is roughly incident on the center of mirror M23.
- 90 Remove mirror M23.

! CAUTION When mirror M23 is removed, the beam will exit the cage cube and continue to an unknown location within the laboratory. Working with open beams at eye level, of any power, is extremely dangerous, and all relevant safety measures and regulations should be followed for this situation.
- 91 Allow the excitation beam to exit the cage cube that mirror M23 is mounted in and propagate to a distant target forming a spot at ‘infinity’.
- 92 Adjust the axial position of L12 to minimize the beam size at ‘infinity’. Despite best efforts, collimating a beam from a high-NA objective is never perfect.
- 93 Reinstall M23.
- 94 Adjust M7 and M23 so that the excitation beam propagates along the center of the 30-mm cage system holding M23, L3 and LS1. Using a cage alignment plate (e.g., Thorlabs CPA1) is recommended.
- 95 The primary excitation path is now aligned; however, the alternative path, used for illuminating a larger area at the sample, is not. To begin alignment, mark the excitation beam positions at the entrance apertures of the mounts for the dichroic mirror DI2 and M22.
- 96 Flip mirrors FM1 and FM2 so that the excitation beam propagates through lenses L10 and L11.
- 97 Remove L10 and L11.
- 98 Using mirrors FM1 and M20, following from Box 1, make the beam straight along the rail supporting mirrors M20 and M21 and parallel to the vertical breadboard surface. FM1 has very limited range of motion, so the excitation beam must be relatively close initially.
- 99 Adjust mirrors M21 and FM2, following the general beam alignment procedure described in Box 1, so that the excitation beam passes the marked positions from Step 95. FM2 has a limited range of motion, so the excitation beam must be relatively close initially.

- 100 Using the marked position at the entrance to the cage cube holding DI2, install lens L11 and adjust its lateral position until the focusing light is incident on the marked position.
- 101 Install lens L10 and adjust the lateral position until the excitation beam is incident on the marked position used in Step 95.

Image separation path alignment ● Timing 2–5 d

- 102 Read the ‘Overview of the procedure’, ‘Image separation path alignment’ subsection, if you have not already done so.
- 103 Install both objective lenses.
- 104 Retract OBJ0 by moving its Z translation stage (N-565) to its highest vertical position, being careful not to crash the objective mount on components above.
- 105 Place a drop of silicone immersion oil on the lower objective (OBJ1).
▲ **Critical step** Without immersion oil between them, transmission through the two opposing objective lenses is limited.
- 106 Engage OBJ0 with OBJ1. That is, move OBJ0 down until it just contacts the immersion oil.
! Caution Use small steps to avoid crashing OBJ0 into OBJ1.
- 107 Hold or mount a card after L1 (collimated path) and carefully step OBJ0 closer to OBJ1 until the collimated alignment laser beam traveling from NPBS to OBJ1 becomes visible.
- 108 Allow the alignment laser beam to reach NPBS and reflect and transmit into the upper portion of the image separation path. Adjust the axial distance between OBJ0 and OBJ1 until a reasonably collimated beam results.
- 109 Insert a pinhole alignment tool into location PH2 and confirm that the alignment laser reflected from the NPBS is incident on the pinhole center and that the alignment laser beam transmitted through the NPBS and cavity is also incident on the center of the pinhole from the opposite side. Slight adjustment to the lateral position of OBJ1 may be required to center the beam arriving from the cavity side.
- 110 Remove the pinhole tool installed in Step 109.
- 111 Fully open apertures AP1 and AP2.
- 112 Install a pinhole alignment tool at locations PH26 and PH9.
- 113 Using the three adjustment screws on mirror M9, walk and tip and/or tilt the alignment beam until it becomes collinear with the pinhole centers at positions PH26 and PH9. Adjusting all three actuators on M9 will translate the mirror surface and, subsequently, shift the beam in one dimension.
- 114 Remove pinhole tools from PH26 and PH9 and install them in positions PH10 and PH11.
- 115 Following from Step 113, adjust mirror M10 until the alignment laser beam is collinear with pinhole centers at PH10 and PH11.
- 116 Remove pinholes PH10 and PH11 and lens L6.
- 117 Install a pinhole tool at location PH19.
- 118 Adjust the three screws in mirror M11 and walk and tip and/or tilt the beam until it is collinear with the center of IR4 and PH19.
- 119 Remove PH19 and install a pinhole tool at location PH14.
- 120 Adjust the tip and/or tilt on PBS0 until the reflected beam hits the center of iris IR8. If PBS0 is mounted correctly (see ‘Equipment setup’), the alignment beam will also pass through the center of PH14. A small error in this step is fine. However, if there is a large misalignment, the mount of PBS0 should be re-seated on its base such that the reflective surface of PBS0 is coincident with the symmetry place of the emission path.
- 121 Remove pinhole PH14 and install a pinhole at location PH17.
- 122 Remove lenses L8 and L9 and prism mirror M17.
- 123 Adjust mirror M15 until the alignment beam is incident on the center of PH17.
- 124 Adjust the tip and/or tilt and piston of M15 until the alignment beam is collinear with IR10 and IR11.
- 125 Adjust M16 so the beam propagating from PBS0 to M16 hits the center of PH17 from the opposite side relative to Step 124.
- 126 Install PH18.
- 127 Adjust the tip and/or tilt and piston (three adjustment screws) of M16 until the reflected beam is collinear with the centers of PH17 and PH18.
- 128 Remove pinholes PH18 and PH17 and lens L7.

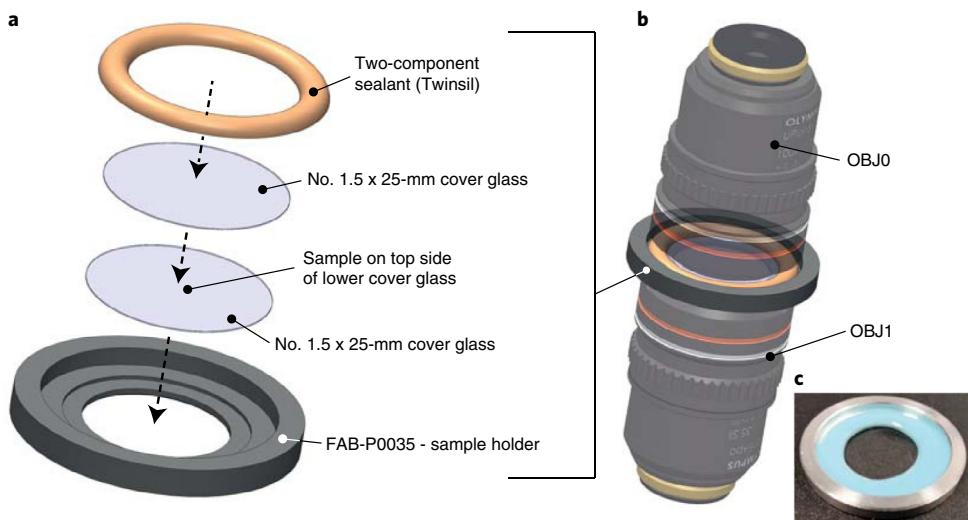


Fig. 15 | Sample mounting in custom holder FAB-P0035. **a**, Exploded view of the sample holder and coverslip assembly. **b**, Resulting geometry of the sample holder located between two opposing objective lenses for imaging. **c**, Photo of mounted sample.

- 129 Check that the alignment beam propagating from M15 and reflected by M16 (to PBS0) passes through the center of IR5. If the alignment beam does not pass the center of IR5, slightly adjust the tip/tilt of M16 until it does. The alignment beam should now propagate from mirror M15 to mirror M16 and be reflected by PBS0 and reach mirror M14.
- 130 Install pinhole PH23.
- 131 Adjust the tip and/or tilt on M14 until the alignment beam hits the center of PH23.
- 132 Install pinhole PH21.
- 133 Adjust the piston and tip/tilt of mirror M14 until the alignment beam is collinear with the centers of PH21 and PH23.
- 134 Remove pinholes PH21 and PH23 and install pinhole PH22.
- 135 Adjust the tip and/or tilt of mirror M13 until the alignment beam hits the center of PH22.
- 136 Install pinhole PH24.
- 137 Adjust the tip and/or tilt and piston of mirror M13 until the alignment beam is collinear with pinholes PH22 and PH24.
- 138 Mirror M12 is the last element to align in the image separation path. However, as the flip mirror used to introduce the alignment laser into the microscope blocks access to this mirror from the NPBS, the position must be adjusted later when imaging a bead sample. To set a reasonable starting position, roughly match the tip, tilt and piston of mirror M12 with that of mirror M9 by eye. Also confirm that the alignment beam reflecting from mirror M13 hits the center of mirror M12.
- 139 Remove any remaining pinholes and open any closed or partially closed irises.
- 140 Install lenses L6, L7, L8 and L9 and mirror M17.
- 141 Hold a card in front of the filter wheel entrance aperture. There should be two vertically arranged spots on the card. The beams arriving from mirrors M15 and M16 should have a small gap. If this is not the case, adjust mirrors M15 and M16 and create a gap.

Sample mounting ● Timing 25–30 min

- 142 Read the relevant portions of the ‘Overview of the procedure’, ‘Sample mounting’ subsection, if you have not already done so. This section is identical for bead and biological samples.
- 143 Make a bead sample according to the ‘Bead samples’ subsection of ‘Reagent setup’).
- 144 Place the sample holder (FAB-P0035) on a clear, level work surface.
- 145 The sample holder contains a Ø25.4-mm recess. Place the sample coverslip into the recessed area with the sample facing up, as shown in Fig. 15a. Ensure that the coverslip is seated in the depression.
- 146 Carefully spread 50–100 µl of immersion medium or imaging buffer onto the sample coverslip evenly.

- 147 Place a second coverslip on top of the sample coverslip, being careful to avoid the introduction of bubbles. This coverslip should also seat into the recessed area mentioned in Step 145. The sample should now be sandwiched between two coverslips with buffer.
- 148 Carefully place a thin, absorbent, lint-free tissue (e.g., a Kimwipe) on top of the coverslip sandwich.
- 149 Place a Thorlabs 19-mm pedestal pillar post (RS075P/M, Thorlabs), base side down, on top of the absorbent tissue. This will apply light pressure to the coverslip sandwich and squeeze any excess liquid out and into the absorbent tissue.
- 150 Remove the pillar post and absorbent tissue, being careful not to remove the coverslip with the tissue.
- 151 Mix equal amounts of two-component silicone adhesive (Picodent, cat. no. 1300 1000) and scoop the result into the open, large end of a 200- μ l pipette tip.
- 152 Apply the two-component silicone adhesive, by pouring from the pipette tip, around the edge of the coverslip such that it creates a seal between the glass and sample holder. As can be seen in Fig. 15b, there is limited space between the two-component silicone and the upper objective for lateral movement. Be careful to keep the two-component adhesive at the edge of the cover glass, or lateral sample movement may be limited.
- 153 Leave the mounted sample to cure in a dark place for \geq 10 min before mounting on the microscope.
▲CRITICAL STEP Some imaging buffers slow the reaction time of the two-component silicone adhesive, requiring more time for the silicone adhesive to cure. Using unequal amounts of the two components will also have an adverse effect on the rigidity and cure time. Be sure to allow enough time for the silicone adhesive to cure.

Loading a sample and setting OBJ1's lateral position ● Timing 60–90 min

- 154 Read the ‘Overview of the procedure’, ‘Loading a sample and setting OBJ1’s lateral position’ subsection, if you have not already done so.
- 155 If not already done, level the sample stage and set its initial Z position following ‘Equipment setup’ above.
- 156 Ensure that the XY piezo stage holding OBJ1 is centered (both axes at 50 μ m).
- 157 Confirm that both shutters in the interference cavity are open. If they are not, open them now.
- 158 If not already done, center the XY sample stage and ensure that it is referenced using PIMikroMove control software (‘Equipment’, ‘Microscope control software’ subsection or ‘Equipment setup’, ‘Device drivers and software’ subsection).
- 159 Retract the upper objective (OBJ0) to its maximum vertical height, being careful not to crash the objective mount with components above.
- 160 Actuate flip mirror FM0 so it is out of the emission path (45° with respect to the optical table surface).
- 161 Raise the sample stage stack using the DC mike motors (M227) so that the sample Z piezo stage insert (where the mounted sample will sit) is 3–5 mm above the lower objective (OBJ1).
!CAUTION Closely monitor the stage stack assembly while in motion to ensure that it does not crash into fixed components above or below.
- 162 Add a drop of silicone oil to the lower objective (OBJ1).
- 163 Insert the mounted sample onto the sample stage. The sample stage has four brass clips (Ted Pella, 16399 and 16399-10) to secure the sample holder in place; do not yet secure the sample with these clips. Leave these clips unused so that as the sample moves closer to OBJ1, it is unlikely to damage the sample or lens should they physically contact each other (crash). Subsequently, it is easy to check if the sample has crashed, as continuing to move the sample stage down will cause the sample to appear to move ‘up’.
- 164 If required, move the sample XY stage so that the sample is centered above the lower objective lens (OBJ1).
- 165 Ensure that the sample Z piezo stage is at its center of the range of motion (50 μ m).
- 166 Slowly step the DC mike motors, so that the sample moves closer to OBJ1, until the sample contacts the lower objective (OBJ1) immersion oil.
- 167 Turn on an appropriate excitation laser and set a relatively high power at the sample.
- 168 Turn on the primary imaging camera and make sure that there are no obstructions in the emission path.

- 169 Select an appropriate emission filter in the filter wheel in front of the imaging camera.
- 170 Set the imaging camera to have no ROI. That is, the full chip is exposed.
- 171 Start live preview on the imaging camera with an exposure time of ~100 ms and ensure that you can see the camera image on your computer screen. At this point, it may be useful to shine a flashlight at OBJ1 while watching the camera readout to ascertain where the bead images will appear on the camera sensor.
- 172 Using the DC mike motors (M227), slowly step the sample closer to the lower objective lens while watching for any signs of beads coming into focus on the imaging camera. A 10–20- μm step size is recommended (smaller step sizes reduce the likelihood that beads will be missed but greatly increase the time required).
- 173 As the DC mike motors bring the sample toward the lower objective (OBJ1), it is likely the beads will move past the focal plane quickly. Watch for any sudden image appearance. When the beads start coming into focus, reduce the DC mike motor step size and roughly focus the beads on the camera without using the piezo Z stage.
- 174 Record the position of the DC mike motors. This position should stay relatively constant across samples.
- 175 Finish focusing the beads using the sample Z piezo stage. Ideally, as the sample has been roughly focused with the DC mike motors, the Z piezo stage will remain close to its center position (50 μm).
- 176 Secure the sample with the small brass clips described in Step 163 and readjust the sample focus if necessary.
- 177 Close the shutter in the emission path of OBJ1 (lower objective).
- 178 Place a drop of silicone immersion oil on the top coverslip of the mounted sample.
- 179 Carefully lower OBJ0 until it makes contact with the immersion oil.
- 180 Record the Z position of OBJ0.
- 181 Carefully step OBJ0 closer to the sample while watching for signs of out-of-focus beads on the imaging camera. A 5–10- μm step size is recommended. This process can take several minutes. OBJ0's Z stage moves quickly, so it is easy to miss the beads passing through the focal plane. Pro tip: it is easy to check if OBJ0 has crashed into the sample by checking the image from OBJ1. If OBJ0 has contacted the sample, the light pressure from OBJ0 will move the sample in Z and thus defocus the image from OBJ1. Retracting OBJ0 will bring the image from OBJ1 back into focus.
- 182 When the beads start to become visible, reduce the step size and bring the beads into focus with OBJ0. If you feel you have missed the beads (stepped them through the focal plane), return OBJ0 to its initial position, recorded in Step 180, and repeat Step 181 with a smaller step size. There is a trade-off between step size, how long it takes to find the beads and the likelihood you miss the beads. It can often take several iterations to focus a bead sample with OBJ0 when first setting up the system.
- 183 With beads in focus, record the Z position of OBJ0. This position is relatively constant across samples. When you switch samples, return OBJ0 to this position as a good initial guess for the focus position.
- 184 Open the shutter for OBJ1 and close the shutter for OBJ0 and make sure that the image from OBJ1 is still in focus.
- 185 Open both shutters in the microscope cavity. You should now see two overlaid images with a potentially large, lateral offset. One image is from OBJ0 and the other from OBJ1. Ideally, the two images will have a small enough lateral offset that they can be merged by moving the lower objective XY piezo stage <10 μm . If this is the case, merge the two images and skip the final (next) step.
- 186 If the images from OBJ0 and OBJ1 have a large lateral offset, or if the offset is so large that features from one image cannot be found in the other, it is helpful to view a larger area. This is easily accomplished using the alternative excitation path to illuminate a large area. Actuate both flip mirrors in the excitation path, FM1 and FM2, such that the excitation beam travels through lenses L10 and L11. It may also be necessary to remove one or both apertures in the image separation path, AP1 and AP2. The XY piezo stage holding OBJ1 (lower objective) can be positioned using two adjustment screws located under the sample stage stack and accessed from the front (sample side) of the microscope. Use these adjusters to move the XY piezo stage until the two images from OBJ0 and OBJ1 are close to overlapping. The lower objective position is very sensitive. Care is required when manually adjusting its lateral position, and getting the images perfectly matched using the adjustment screws is not easy. Finally, merge the images from OBJ0 and OBJ1 using the lower objective (OBJ1) XY piezo stage. If removed, install apertures AP1 and AP2 and return the excitation path to its original condition.

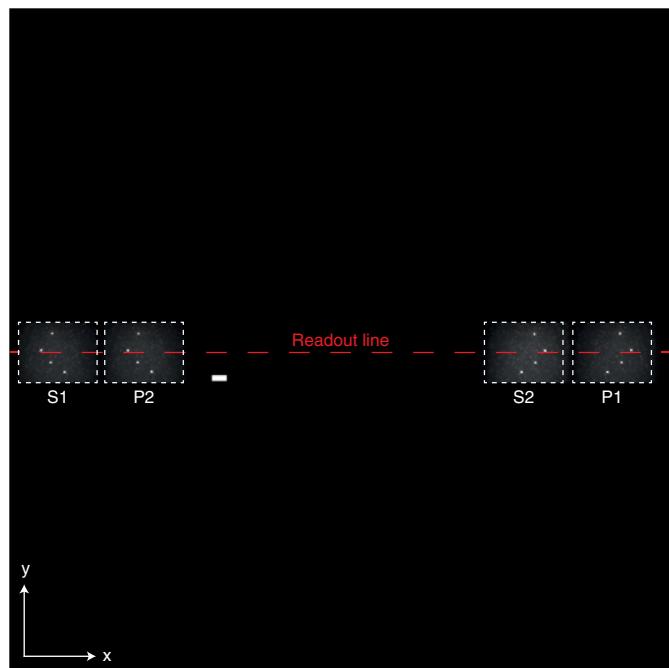


Fig. 16 | Image layout on data-acquisition camera. Fluorescent bead images on the primary imaging camera arranged in a line along the X-dimension. Two sets of images are visible with a gap between them. Images S2 and P1 are flipped relative to S1 and P2. The scale bar is 5 μm .

Image setup and alignment ● Timing 2–4 h

- 187 Make a dense bead sample following the ‘Bead samples’ subsection of ‘Reagent setup’ and mount it following Steps 144–153.
- 188 Switch on appropriate excitation sources for the beads being used.
- 189 Select an appropriate emission filter in the filter wheel in front of the imaging camera.
- 190 Bring the beads into focus on the imaging camera with both objective lenses. Four images should be visible on the camera.
- 191 Set the camera to live preview mode (continuous running) with ~100-ms exposure time.
- 192 Ensure that the beads are appropriately focused with both OBJ0 and OBJ1 and that the images from both objectives are laterally matched.
- 193 Close the shutter corresponding to OBJ0 (upper) and open the shutter for OB1 (lower).
- 194 The effective camera pixel size is ~130 nm. Thus, a $25 \times 25\text{-}\mu\text{m}$ illumination area will correspond to a 200×200 -pixel area on the imaging camera. Adjust the aperture AP0 in the excitation path until all four images have a 200×200 image format. Be sure to close the aperture around the center of the field of view. Restricting the illumination area in this way minimizes photobleaching features outside the imaging area.
- 195 Close the blades of aperture AP2, in the lower portion of the image separation path, until the aperture matches the boundaries set in the previous step. That is, AP2 should now match AP0 on the imaging camera. With only OBJ1 ‘on’ (shutter SH1 open), AP2 will have an apparent effect on only two of the four images visible on the camera.
- 196 Toggle the shutters (open SH0 and close SH1).
- 197 Repeat Step 195 with aperture AP1 (upper portion of the image separation path).
- 198 The images on the camera should, roughly, be arranged in a line with two image pairs (one pair coming from mirror M15 and the other from mirror M16, top and bottom). Using mirrors M15 and M16, move the four images along the Y-dimension of Fig. 16 so that they are centered on the camera sensor. M15 will move one pair of images together while M16 will move the other pair.
- 199 Set a $256 \times 2,048$ (full chip) ROI on the camera, centered on the camera sensor and oriented so that the central readout line of the camera (as described in ‘Equipment setup’, ‘Primary imaging camera orientation’ subsection) is parallel to the long dimension of the ROI. The central shutter line of the

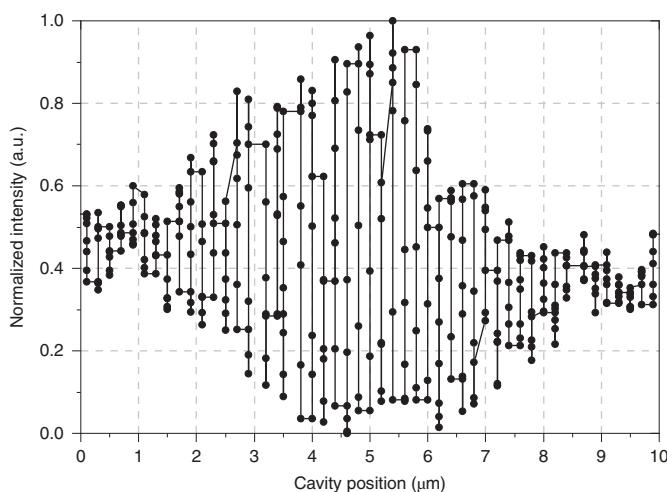


Fig. 17 | Example interference scan data. Example interference data when scanning a single bead by translating the stage stack through the central interference position.

sensor can typically be seen when the camera is in live preview mode with the sensor cooler off and no light reaches the camera.

- 200 Use mirrors M15 and M16 and adjust the Y-position of the four images so that they form a line along the X-dimension in Fig. 16 and are centered about the Y-dimension of the ROI.
- 201 If either set of images are overlapping or have less than ~10 pixels separating them, adjust mirror M11 or M14 to increase the distance between the two images.
- 202 Using mirror M15, bring the upper set of images as close to the center of the ROI, along the X-dimension, as possible without clipping or distortion.
- 203 Repeat Step 202 with the lower set of images and mirror M16. Irises IR2 to IR14 may be used to further check that the image positions match the design locations.
- 204 Reduce the camera ROI size to $200 \times N$, where N is the minimum number of pixels needed to keep all four images within the ROI.

Initial interference location ● Timing 1–5 d

▲ **CRITICAL** Ensure that the camera is using liquid cooling and that the internal fan is switched off before attempting to locate interference. Vibration from the camera fan will make interference difficult or impossible to locate.

- 205 Make a medium-density bead sample following ‘Reagent setup’.
- 206 Mount the sample from Step 205 on to the microscope as described in Steps 144–153.
- 207 Bring the bead sample into focus, on the imaging camera, with both objective lenses.
- 208 Laterally align OBJ1 (lower) with OBJ0 (upper) such that the images from both objectives are merged.
- 209 Set the imaging camera exposure to 10 ms (100 frames per second (FPS)).
- 210 Set the LS-50 translation stage speed to 0.001 mm/s.
- 211 Record the current LS-50 positions.
- 212 Ensure that both interference cavity shutters (SH0 and SH1) are open.
- 213 Set the imaging camera to live preview (continuous run) mode.
- 214 Choose a scan direction (up or down) and initiate a small (50-μm) step in that direction.
- 215 While the LS-50 stages are moving, watch the bead images closely. When the path length difference between the upper and lower cavity arms is smaller than the coherence length, a strong and visually apparent fluctuation in bead image intensity will be observed across the field of view. If you reach this point, note the scan position and stop the stage movement. If you do not observe interference, continue to the next step. If interference has been observed, skip to Step 217.
- 216 (Optional) After the LS-50 stages have finished the 50-μm step and interference has not been observed, check the sample focus, with both objectives, and the objective lateral alignment. As necessary, refocus and laterally align the objectives. Start another 50-μm step with the LS-50 stages,

in the same direction as chosen in Step 214, and repeat Step 215. If the LS-50 stages have moved 2 mm in one direction with no observable interference, return them to the position recorded in Step 211 and then repeat Steps 214 and 215, this time starting your scan in the opposite direction relative to that initially chosen. If interference has been observed, continue to the next step.

? TROUBLESHOOTING

- 217 Once the interference location has been found, an additional scan should be performed to locate the maximum interference. Move the LS-50 stages to the edge of the interference zone.
- 218 Step the LS-50 stages 20 µm back through the interference zone and observe the position resulting in the largest contrast between constructive and destructive interference, as shown in Fig. 17. Record this position. From sample to sample and day to day, the interference location will be within ±30 µm of this position, provided the system is set up in a temperature-controlled and vibration-free environment.
- 219 Move the LS-50 stages to the location with maximal interference.

DM calibration and centering ● Timing 1-2 d

- 220 Calibrate both DM0 and DM1 following the procedure from Antonello et al.²⁴ as referenced in ‘Equipment setup’. This will produce a mirror flat and Zernike modes needed for creating a system flat and applying astigmatism.
- 221 Prepare a sparse bead sample (see ‘Reagent setup’).
- 222 Mount the bead sample on the microscope as described in Steps 144–153 and bring the beads into focus with both objectives.
- 223 Apply the respective mirror flats generated in Step 220 to both DM0 and DM1.
- 224 Laterally align OBJ1 with OBJ0 such that the images from both lenses are merged.
- 225 Close the shutter in the lower cavity path (SH0) so that the visible image is only from OBJ0 and DM0.
- 226 Select a bead that is spatially separated from close neighbors and mark its in-focus center position.
- 227 Move the sample Z stage 1–2 µm up or down to defocus the marked bead (increasing the excitation power may be necessary to visualize the defocused bead).
- 228 Apply +2 radians of astigmatism to DM0. The defocused marked bead should now appear to be a long thin line.
- 229 Laterally translate DM0 along one axis until the defocused line image of the bead matches the center position marked in Step 226. It is likely you will need to move both the X and Y DM translators to determine the correct axis to move.
- 230 Invert the astigmatism mode value (sign) and move the axis not used in Step 229 until the defocused bead image (line) is coincident with the position marked in Step 226.
- 231 Apply a symmetrical mirror mode to the DM and move the bead through the microscope focus. Make sure that the PSF is still symmetric.
- 232 Close shutter SH0 and open shutter SH1.
- 233 Repeat Steps 220–232 for DM1.

Phase adjustment across the field of view ● Timing 1-2 h

- 234 Visualizing interference fringes with beads requires a very high density. Prepare a high-density bead sample (see ‘Reagent setup’).
- 235 Mount the sample from Step 235 on the microscope as described in Steps 144–153.
- 236 Bring the beads into focus with both objectives.
- 237 Apply mirror flats to both DMs.
- 238 Laterally align OBJ1 with OBJ0 such that the images from both lenses are merged.
- 239 With both cavity shutters open, adjust the stage stack position to maximize interference (following Steps 217–219).
- 240 Remove one of the apertures (AP1 or AP2) from the emission path to enlarge the field of view. Initially, using a larger field of view will help get a sense of the interference fringe pattern.
- 241 Set the camera to full chip (no ROI) to see the larger field of view.
- 242 Actuate flip mirrors FM1 and FM2 in the excitation path to illuminate a larger area in the sample.
- 243 While observing the interference fringes, choose one axis (servo motor) of the NPBS and pick an initial direction. Take a small step in that direction with that motor and observe the change in fringe frequency (spacing of interference peak positions). If no change is observed, take another small step until a change in the fringe frequency is observed.

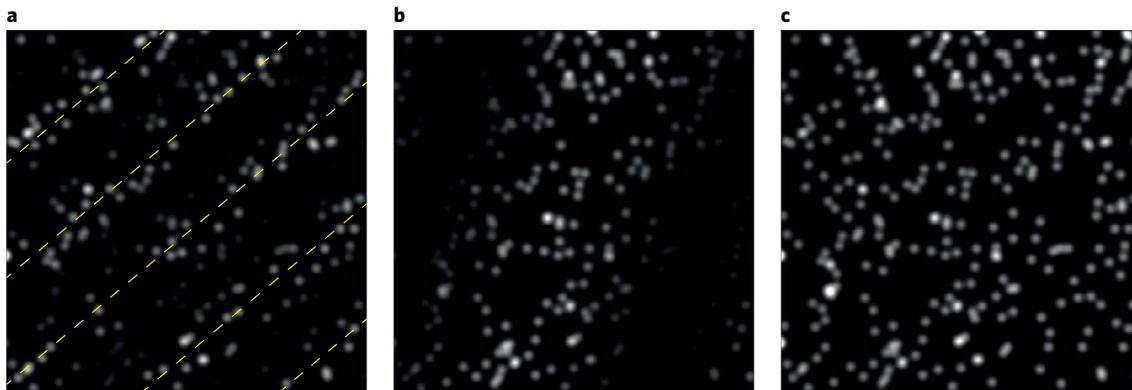


Fig. 18 | Interference variation across the field of view. After initial setup, interference will vary across the ~20 × 20-μm field of view as shown in **a** when viewed with a high-density bead sample (simulated images). Yellow lines in **a** guide the eye in seeing the constructive interference fringes. As the OBJ1 and the NPBS are iteratively adjusted, the number of fringes will be reduced as shown in **b**. After careful adjustment, the interference phase across the field of view can be made reasonably uniform as shown in **c**.

- 244 If the servo motor movement from Step 243 resulted in the fringe frequency going down, continue stepping that motor in the same direction. If the frequency went up, reverse direction and continue stepping until the fringe frequency is reduced. As the servo motor is stepped (tipping or tilting the NPBS), the images from OBJ0 and OBJ1 will become laterally misaligned. It is necessary to periodically laterally realign OBJ0 and OBJ1 during this process.
- 245 As the servo motor chosen in Step 243 is moved, the interference fringe frequency will be reduced, and the angle of the fringes will appear to change. When the fringes reach their minimum, from adjusting this servo motor, the lines of constructive interference (highlighted with yellow dashed lines in Fig. 18a) will become oriented either vertically or horizontally. When this happens, switch to the other NPBS axis (servo motor).
- ▲ **Critical step** As the number of fringes is reduced to 1 or 2 (see Fig. 18b for example), it becomes necessary to take smaller steps with the servo motors controlling the NPBS tip/tilt. However, it is very difficult to fully remove any phase variation across the field of view because of two complicating factors. First, the servo motors have some backlash, and changing direction when taking small steps will result in an unpredictable step size. Second, the rotation and goniometer stages holding the NPBS have some hysteresis. In combination, these problems make it difficult to systematically remove all phase variation across the field of view.
- 246 Step the second axis until the interference fringe frequency is reduced while periodically laterally realigning OBJ0 and OBJ1. As the number of fringes is reduced to 1 or 2 (Fig. 18b), it is useful to return the excitation path to the configuration for illuminating a small area and zoom in on one of the four images for closer inspection.
- 247 Continue alternately adjusting the tip and tilt of the NPBS until the fringes are minimized as in Fig. 18c.
- 248 Reinstall the aperture removed in Step 240.

Quartz wedge calibration ● Timing 5–10 h

- 249 Prepare a multicolor bead sample (1:100 (vol/vol) dilution from stock) as described in ‘Reagent setup’.
- 250 Power on the controller for the motor that controls the position of the quartz wedge pair in the upper cavity beam path (W0) and open the appropriate software (e.g., Thorlabs’ Kinesis) for computer control.
- 251 Ensure that, visually, the quartz and BK7 wedges are close to their design positions (no obvious visual displacement between the moveable and fixed wedge).
- 252 Two emission filters must be installed in the imaging camera filter wheel (FW, Fig. 10). If not already installed, install a 700/75-nm far-red emission filter (Chroma, ET700/75m or equivalent) and a 600/52-nm orange emission filter (Semrock, FF01-600/52-25 or equivalent).
- 253 Select the 700/75-nm far-red filter.
- 254 Mount the sample prepared in Step 249 on the microscope as described in Steps 144–153 and bring the beads into focus with both objective lenses using a far-red excitation laser.

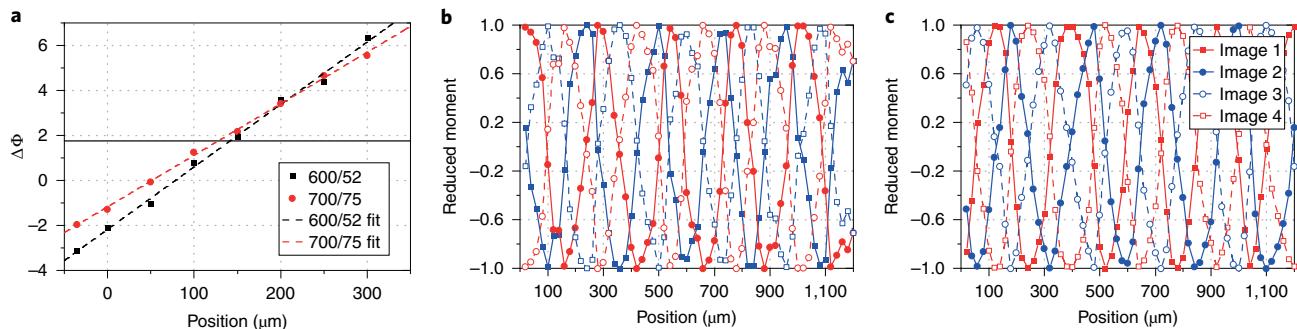


Fig. 19 | Example quartz wedge calibration data for setting the phase between the four interference images. The quartz wedge motor position versus phase for both color channels is shown in **a**, while the resulting reduced moments for the 600/52 filter and 700/75 filter are shown in **b** and **c**, respectively.

- 255 Apply mirror flats to both DMs.
- 256 Laterally align OBJ1 with OBJ0 such that the images from both lenses are merged.
- 257 Close shutter SH0 and collect a single image with the lower objective lens only (this will be needed to generate a transformation between all four images during data analysis).
- 258 Open the data analysis software.
- 259 In the data analysis software opened in the previous step, use the ‘Channel Alignment’ function and select the image collected in Step 257. This will generate the transformation used to register the four images and only needs to be done once.
- 260 Open shutter SH0.
- 261 With both cavity shutters open, adjust the stage stack position to maximize interference (following Steps 217–219).
- 262 Pick a step direction and step the motor controlling the quartz wedge pair (W0) 100 μm from its initial position and note the direction of the step (positive or negative).
- ▲CRITICAL STEP** Most motors used for this application (wedge positioning) will have some backlash, making it difficult to return to a previous position. Thus, several iterations of this procedure may be necessary before the correct position is determined.
- 263 Repeat Step 256 if any lateral misalignment is observed between the merged images.
- 264 Move the sample Z stage down 1 μm .
- 265 Collect a Z stack image with a 300-ms camera exposure time, a 20-nm step size and 101 steps.
- 266 Change the filter from the 700/75-nm far-red filter to the 600/52-nm orange filter (changing the excitation laser may be necessary) and ensure that the beads are well focused in the second color channel.
- 267 Repeat Step 265 (take a second Z stack with the same parameters, changing only the filter).
- 268 Using the data analysis software opened in Step 258, run the ‘Find Phaseshift’ function on the Z stacks collected in Steps 265 and 267. Each time the ‘Find Phaseshift’ function is run, the phase difference between Δs and Δp will be reported. Example phase data are shown in Fig. 19a.
- 269 Record the phase difference for the far-red Z stack and the orange Z stack and the current wedge (motor) position.
- 270 Step the motor controlling the quartz wedge position a small amount (50 μm) in the direction opposite to that initially used in Step 262.
- 271 Repeat Steps 265–269 and note the phase difference of each color channel at the new wedge (motor) position.
- 272 Repeat Steps 270 and 271. That is, continue to move the wedge (motor) in small increments while recording the phase difference for each color channel. In this way, you will build a table with wedge positions versus phase difference (for both color channels) with the goal of finding a wedge (motor) position that results in both the far-red and orange channels having a phase difference close to $\pi/2$ (it will not be possible to have both channels at exactly $\pi/2$). Example data collected during this process are shown in Fig. 19b.
- 273 Continue repeating Step 272 until a wedge position results in both color channels having a phase difference close to $\pi/2$. It may be necessary to return to the initial wedge position (Step 262) and iteratively move the wedge in the opposite direction.

DM system flat and astigmatism calibration ● **Timing** 30–60 min

- 274 Apply their respective mirror flats to DM0 and DM1 following from Step 220.
- 275 Prepare a sparse bead sample (see ‘Reagent setup’) and mount it on the microscope as described in Steps 144–153. Bring the beads into focus with both objectives.
- 276 Laterally align OBJ1 with OBJ0 so that the images from both lenses are merged.
- 277 Close the upper shutter (SH0) and open the lower shutter (SH1). The bead should now be imaged by the lower objective (OBJ1) only.
- 278 Adjust the sample Z position until the best focus for OBJ1 is visually achieved.
- 279 To fine-tune the focus, scan the defocus mode (mode 4) and plot the image metric versus mode amount. Fit this data and locate the peak position. Please note that this is an automated process in the DM control software. If the fitted peak corresponds to a mode amount of approximately zero, the sample is well focused; move to the next step. If the fitted peak is not at zero, refocus the beads and repeat this step.
- 280 Scan DM1 mirror modes for primary coma (modes 8 and 9), primary spherical (mode 13) and astigmatism (modes 3 and 5) to compensate for lower-order aberrations in the system (scanning modes are automated within the microscope control software). Typically, these modes will result in the largest improvement to the PSF. When applying vertical (mode 5) and oblique (mode 3) astigmatism, check if the defocused PSF (which will form a vertical or horizontal line for mode 5) is straight (up and down, left and right) and symmetric and similarly at 45° for mode 3. If the PSF requires further improvement, scan higher-order coma (modes 21 and 22) and higher-order spherical (mode 28).
- 281 Once the PSF looks reasonable, save the resulting DM pattern. This is the system flat for DM1.
- ? TROUBLESHOOTING**
- 282 Close the lower cavity shutter (SH1) and open the upper shutter (SH0).
- 283 Repeat Steps 278–281 for DM0.
- 284 With SH0 open and SH1 closed (imaging from top objective, OBJ0, only), defocus the bead sample 600 nm (above or below the focal plane).
- 285 Apply +0.8 radians of vertical astigmatism (mode 5) to DM0. The PSF should now appear as either a vertical or horizontal line (depending on the defocus direction).
- ▲ CRITICAL STEP** For the analysis program to work correctly, DM0 must have a positive astigmatism value and DM1 must be negative.
- 286 Note the orientation (vertical or horizontal) and length of the defocused PSF.
- 287 Close SH0 and open SH1.
- 288 Apply –0.8 radians of vertical astigmatism (mode 5) to DM1. The PSF from the lower objective should now appear as a vertical or horizontal line.
- 289 Adjust the amount of mode 5 on DM1 until the defocused PSF from OBJ1 matches the size and orientation of the defocused PSF from OBJ0.
- 290 If application of mode 5 does not result in a vertically or horizontally oriented PSF (slight tilt angle), adjust the amount of mode 3 (oblique astigmatism) in small steps to change the PSF orientation.
- 291 With the bead sample still defocused by 600 nm, adjust the amount of astigmatism (mode 5) on DM1 until the ratio of the PSF long axis to short axis (σ_x/σ_y) is between 3 and 3.5 when defocused on one side of the focal plane and between 0.29 and 0.33 when defocused on the other. Record this value.
- 292 Apply the opposite sign but same magnitude to mode 5 of DM0. The respective values for astigmatism (mode 5) on DM0 and DM1 must be applied when imaging for the image reconstruction to work (these values can be saved and toggled on and off within the DM control software). In this way, the astigmatic PSF from OBJ0 and OBJ1 have the same shape on either side of the focal plane, and the elongated PSF shapes are symmetric across the focal plane in Z (i.e., σ_y/σ_x at –600-nm defocus is equal to σ_x/σ_y at +600-nm defocus).
- 293 Confirm that the PSF is symmetric across the focus by moving the sample above and below the focal plane for each objective lens. If the PSF shapes, when defocused, are different on either side of the focus position, rescan the spherical aberration modes (13 and 28) until they become symmetric.
- 294 Move the sample to the position of maximal interference and check the PSF with astigmatism off. Bead images of the same shape with different intensities across the four channels should be observed. Move the sample up and down in small steps (100–200 nm) to observe the intensity variation. The PSF should remain the same for all imaging channels.

Box 2 | Analysis software center positions ● **Timing** 5–20 min

The analysis software GUI has four numeric ‘Channel Center’ input boxes. These correspond to the horizontal image positions within the camera ROI set in Step 204, moving from left to right. For the software ‘Channel Alignment’ function to work, the horizontal center positions of the four phase images must be entered. To ascertain an initial guess for the center positions for each image, open the Hamamatsu DCIMG image collected in Step 301 and determine the X coordinate for each of the four images, from left to right. Enter these values into the ‘Channel Center’ boxes, from left to right.

After finishing Step 302, a file named ‘align_642_FMTtransform_currentDate.mat’ will be saved to disk. Open this file and check the value of the variable named ‘trans_all’. The first column contains the horizontal shift between images 1, 2, 3 and 4 relative to image 1 based on the user input values from the GUI (‘Channel Center’ input boxes). The shift values should all be <1 (pixel). Based on the values in ‘trans_all’, adjust the user input center position values and run the ‘Channel Alignment’ function again. Check the new values in ‘trans_all’ and repeat the process until the shift values are <1.

Default center positions are stored in the analysis program file named ‘SMS_4Pi.m’ within the function ‘SMS_4pi_OpeningFcn’. After the best center positions are determined, modify this function to load your center positions by default.

Image registration ● **Timing** 1–5 min

- 295 Prepare a medium bead sample (see ‘Reagent setup’).
 - 296 Mount the sample on the microscope as described in Steps 144–153.
 - 297 Focus the beads with two objectives and overlay the images.
 - 298 Locate a field of view with no less than 10 beads.
 - 299 Set the camera exposure time to 0.3 s and the number of frames to collect as 10.
 - 300 Adjust the laser power so that the bead image has a high signal-to-noise ratio.
 - 301 Record 10 camera frames.
 - 302 Using the analysis software, run the ‘Channel Alignment’ function on the images recorded in Step 301. Take note of the channel center information described in Box 2. The software will apply affine transformations to the images from the four channels, and if successful, a ‘.mat’ file will be generated with calibration information for later use.
- ▲CRITICAL STEP** The ‘Channel Alignment’ function within the analysis software will display four merged images. In each image, channel 1 is shown overlaid with channels 1–4 after transformation. Visually confirm that the transformation has resulted in a good match between all channels with channel 1.

Phase calibration ● **Timing** 5–20 min

- 303 Prepare a low-density bead sample (see ‘Reagent setup’) and mount it on the microscope as described in Steps 144–154.
 - 304 Bring the bead sample into focus with both objective lenses and laterally align the resulting images on the imaging camera.
 - 305 Scan the LS-50 stages and adjust the cavity for maximum interference.
 - 306 Refocus and align both objective lenses.
 - 307 Apply system flats to DM0 and DM1, respectively.
 - 308 Apply astigmatism as determined in Steps 291 and 292.
 - 309 Move the sample Z stage down 0.6 μm.
 - 310 Set up a Z-stack acquisition with an exposure time of 0.3 s/frame, Z step size of 0.02 μm and 61 steps.
 - 311 Adjust the excitation laser power for high signal-to-noise ratio images.
 - 312 Start the Z stack and observe data collection. Example bead images across this Z range are shown in Fig. 20. Specifically, Fig. 20a shows an in-focus bead without astigmatism while b, c and d show the same bead with astigmatism applied at –600, 0 and 600 nm, respectively.
 - 313 After Z-stack acquisition has finished, run the MATLAB analysis program (<https://github.com/4Pi-SMS-consortium/4Pi-SMS-analysis>) and use the ‘Find Phaseshift’ function on the Z-stack collected in Step 312. You will be required to manually select a single bead.
 - 314 As the ‘Find Phaseshift’ function runs, several plots will be displayed. These show the reduced moment versus step position³ of the four channels (Fig. 21a) and unwrapped phase versus Z position (Fig. 21b). The value of Δθ is reported in the MATLAB command window.
- ▲CRITICAL STEP** Confirm that the value of Δθ is close to ±1.57 and that the unwrapped phase versus Z position (Fig. 21b) displays a straight line as expected. If results do not look like the example data shown in Fig. 21, select another bead from the same image stack or repeat the experiment.

Objective lock setup ● **Timing** 4–8 h

- 315 Prepare a low-density bead sample (see ‘Reagent setup’) and mount it on the microscope as described in Steps 144–153.
- 316 Overlay the images from OBJ0 and OBJ1.
- 317 Check that M7 in the emission path allows the transmitted excitation beam to pass through the center of the mount of M23 (Fig. 14).
- 318 Install the 940-nm NIR laser fiber (LP940-SF30, Thorlabs) on the Z-Axis Translation Mount (SM1Z, LS1). Install lens L13 (AC254-060-B-ML, Thorlabs) in the XY translation mount (CXY1, Thorlabs) and adjust its axial position to collimate the NIR beam with an NIR viewing card over a large distance.
- 319 Adjust M23 to allow the NIR beam to hit the center back aperture of the upper objective. The NIR beam will come out of the lower objective and travel in the opposite direction relative to the excitation beam along the same path.

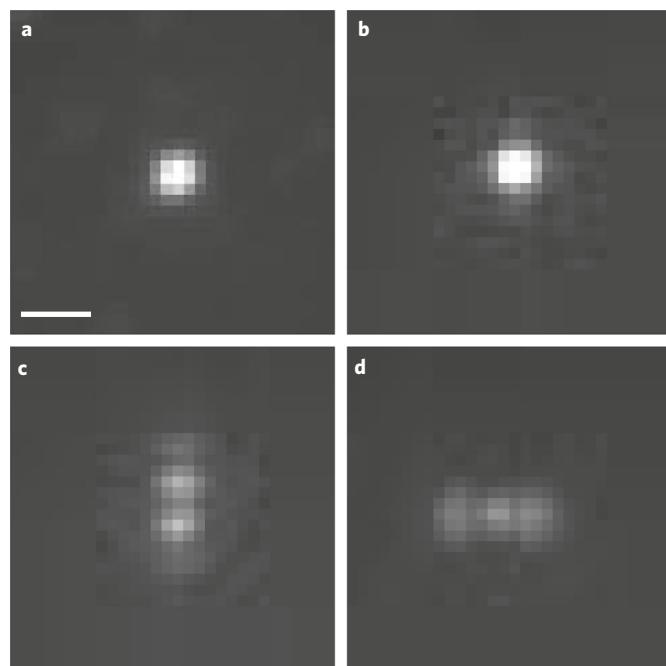


Fig. 20 | Fluorescent bead with and without astigmatism above and below the focal plane. **a**, Image of a single fluorescent bead at the focal plane without application of astigmatism. The same fluorescent bead is shown in **b** with astigmatism applied while in focus and defocused by -600 nm (**c**) and $+600$ nm (**d**), respectively. The scale bar is $1\text{ }\mu\text{m}$.

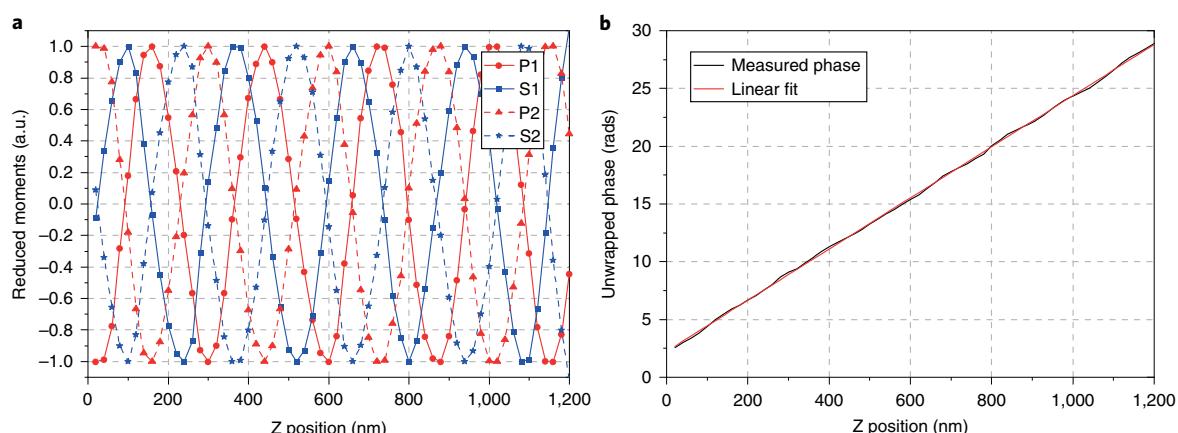


Fig. 21 | Reduced moment versus Z position and phase calibration data example. **a**, Reduced moment for the four phase images. **b**, Unwrapped phase versus sample Z position fit to a linear function.

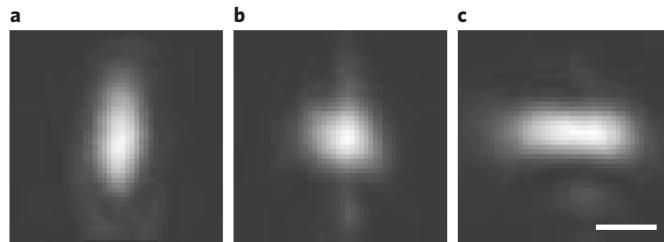


Fig. 22 | Objective lock NIR laser focus. A 940-nm NIR laser is used to track the relative position of the two objective lenses. After passing through OBJ1 and being collected by OBJ0, the beam passes a cylindrical lens and is focused on a camera. When the object's axis separation changes (defocused) by $-1\text{ }\mu\text{m}$, the NIR laser spot appears as in **a** on the camera. When the objectives are 'in focus', the spot appears as in **b**, and when they are axially separated by $+1\text{ }\mu\text{m}$, the NIR spot appears as in **c**. The scale bar is $20\text{ }\mu\text{m}$.

- 320 Insert a short-pass filter at position DI2 (DMSP805L, Semrock) into the filter cube (LC6W, Thorlabs) after M22 and reflect the NIR beam up toward M24.
- 321 Install mirror M24 and then the camera (DCC1545M, Thorlabs) at position CAM1.
- 322 Adjust M24 so that the 940-nm NIR beam hits the center of CAM1.
- 323 Remove CAM1.
- 324 Insert the cylindrical lens CY0 (LJ1144RM-B, Thorlabs) between M24 and CAM1 to introduce astigmatism to the focus so that the focus is line shaped when the focal planes of the two objective lenses are no longer coincident.
- 325 Reinstall CAM1. Figure 22 shows the shape of the laser beam when the two focal planes of the objectives are matched and when they are defocused by $-1\text{ }\mu\text{m}$ and $+1\text{ }\mu\text{m}$. Monitoring the position and shape of the focused NIR beam on CAM1 allows the change in relative position between OBJ0 and OBJ1 to be tracked.
- 326 The control software includes a module for objective lock operation (<https://github.com/Gurdon-Super-Res-Lab/Microscope-Control/tree/master/Microscope%20Modules/4Pi%20Objective%20Lock>). Following the associated software instructions, run this module.
- 327 Select an ROI ($<100 \times 100$ pixel) around the focused beam on CAM1.
- 328 Set an appropriate drift threshold (e.g., 10 nm for XY) and monitor the target and fit position of the focus to make sure that the values are reasonable.
- 329 Select appropriate proportional, integral and derivative (PID) control values (reasonable default values should already be loaded) and activate the objective lock to confirm that it is working as expected.

Mount a cell sample ● Timing 15–30 min

- 330 Culture appropriate cells on a no. 1.5H \times Ø25-mm round coverslip and prepare (label) the cell sample for imaging (see 'Reagent setup').
- 331 Mount the sample coverslip into the sample holder with the cell side facing up according to Steps 144–153.
- 332 Add a small amount of diluted crimson beads (e.g., 20 μl of 1:50,000 (vol/vol) dilution from stock) to the top of the cell sample. The sparse beads in the cell sample are required for PSF optimization and alignment and while imaging.
- 333 After 2 min, wash away the beads with PBS.
- 334 Evenly spread 100 μl of the imaging buffer on the sample coverslip and then place a clean coverslip on top.
 - ▲ **Critical Step** Avoid bubbles trapped between the two coverslips. If bubbles are present, remove the top coverslip and repeat Step 334.
- 335 Carefully place a thin, absorbent, lint-free tissue (e.g., a Kimwipe) on top of the coverslip sandwich to absorb excess imaging buffer.
- 336 Seal the cell samples with the two-component silicone glue (see Steps 150–152).
- 337 After the silicone glue hardens (typically 20–30 min), load the sample onto the microscope. The imaging buffer typically allows imaging for 8–10 h.
- 338 Find a bead in the cell sample.
- 339 Focus the bead found in Step 338 with both objectives and laterally align both objective lenses.

- 340 Scan the cavity for interference and move to the maximum interference position.
- 341 Add astigmatism to both the upper DM and the lower DM with the amount determined in Steps 291 and 292.
- 342 Acquire a Z-stack of the bead as in Step 310. Use this Z-stack to generate a phase calibration as in Step 313.

Imaging the cell sample ● Timing 10–60 min

- 343 Lock the objective positions (Step 329).
- 344 Set imaging parameters. Typical imaging parameters for biological samples are given in the table below. Quality image examples are given in ‘Anticipated results’.

Imaging parameters	Typical values
Exposure time	5–10 ms
Excitation laser intensity	5–15 kW/cm ²
Frame number	We use 3,000 frames per cycle (image) to limit file size for the analysis software. 60–120 cycles are usually enough for ‘thin’ samples (no Z stacks) or a thin volume in thicker samples without need for Z stacks.
Step size for Z stacks	500 nm

? TROUBLESHOOTING

- 345 Locate a suitable field of view for imaging.
- 346 Increase the excitation laser power to push the molecules into the dark state. Depending on the sample, it may take from 2 to 30 s before the density of active molecules reaches a low enough level.
- 347 Start data acquisition. As needed, adjust the 405-nm laser intensity to keep the blinking density relatively constant.

Data analysis ● Timing 1–3 h

- 348 Create a new folder on the analysis computer called ‘Cell_Data’, and inside of ‘Cell_Data’, create a folder called ‘Images’, a folder called ‘Registration’ and a folder called ‘Phase_Calibration’.
- 349 Transfer the data (all Hamamatsu DCIMG images) collected in Step 347 to the folder named ‘Images’ on the analysis computer created in Step 348.
- 350 Copy the image (single DCIMG file) collected in Step 301 (Image registration) to the folder named ‘Registration’ created in Step 348.
- 351 Copy the data (multiple DCIMG files) collected in Step 312 (Phase calibration) to the folder named ‘Phase_Calibration’ created in Step 348.
- 352 Run the analysis software referenced in ‘Experimental design’. When the analysis program GUI appears, click the ‘Main Folder’ button and select the folder named ‘Cell_Data’ that was created in Step 348.
- 353 Click the ‘Channel Alignment’ button in the analysis software GUI and select the DCIMG file from Step 350. Allow time for the channel alignment routine to run. This will generate a transformation file as described in ‘Experimental design’, ‘Data analysis’ subsection. Be sure to use the image center values described in Box 2.
- 354 Click the ‘Find Phaseshift’ button in the analysis software GUI and browse to the ‘Phase_Calibration’ folder created in Step 348. Select all the DCIMG files inside the ‘Phase_Calibration’ folder (in Windows, press Ctrl+A to select all the DCIMG files) and click ‘Okay’. Allow time for the phase calibration to run. This will generate a phase calibration file as described in ‘Experimental design’, ‘Data analysis’ subsection.
- 355 The data set sitting in the ‘Images’ folder will appear in a list box below the ‘Main Folder’ button in the analysis software GUI. Select this data set in the list box and press the ‘Get Positions’ button. Allow time for this analysis step to run (hours).
- 356 After Step 355 has finished, switch to the ‘Reconstruct’ button (upper right) and select your position data set from the list box on the left-hand side. Click the ‘Reconstruct’ button at the bottom right. Apply drift correction if required. Localization information is saved as comma-separated values in a text file.

? TROUBLESHOOTING

Troubleshooting

As this is a complex optomechanical system that also requires properly labeled samples, there are many potential pitfalls that could lead to a non-functioning microscope or non-optimal results. Table 1 lists several known issues that are common, to our knowledge, and unique to the 4Pi configuration, with some easy solutions (Figs. 23 and 24). We encourage readers to contact us if unknown issues are seen or more difficult solutions are required. Furthermore, the corresponding authors are available to address any technical questions upon reasonable request.

Table 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
216	Inability to achieve interference	The cavity arms are unequal in length	Additional length can be added to either the upper or lower interferometric arm by adding glass (e.g., adding a piece of glass slide (1-mm thickness) to one arm will increase its path length ~300 μm and will shift the interference peak position by 150 μm in the same direction). Also check the axial locations of the DMs in the mounts
		Interference fringes are varying too fast The interference contrast is too low	Make sure you are scanning the cavity slowly enough Use bright beads or long exposures to achieve a good single-to-noise ratio
281	Heavily distorted PSF	DMs are sensitive devices that have numerous actuators. Even a single actuator's fault on the DM will lead to distortion in the PSF	Regular checks on the membrane are encouraged. A quick check can be done visually by inspecting the reflected beam off the DMs using the alignment laser. Where there are faulty actuators, it is likely that the reflected beam is not Gaussian shaped, which can be easily seen by visual check. A full inspection can be done using our dedicated compact interferometer ¹⁹ . A 2D interferogram (Fig. 24) obtained from such a device presents peaks and troughs at the locations of the dead actuators. A DM dummy flat mirror can be ordered from the supplier and might help in setting up the instrument and identifying DM faults
344	Low localization precision	The vertical breadboard of the microscope can make the system prone to picking up vibrations from the environment. This will lead to variations of the sample position as well as to variations in the optical path lengths in the cavity. Both might result in a loss in localization precision	Lateral vibrations will produce smeared-out localizations. Vibrations impairing the axial localization precision manifest as shifts in the intensity between the four quadrants. To check for potential vibrations, take images of sparse beads in a single plane at high frame rates (e.g., 1,000 Hz)
		Vibration sources such as refrigerators, air control units, centrifuges and electrical inverters	Check the anticorrelation between the intensities of different quadrants (Fig. 23a) to see the effect on axial localization precision. The power spectral density (Fig. 23b) can be used to find the dominant frequencies of the disturbing vibrations (up to half the imaging rate). Find the source of vibration and remove or relocate the source
		Impaired damping on the optical table	Record audio spectra near suspicious locations using, for example, a Shure SM57 microphone with Scarlett Solo USB sound card. Plotting the spectra over time in a kymograph-like manner (Fig. 23c) can reveal fingerprint-like behavior of vibration sources that might be moved or damped to mitigate the effect on the microscope vibrations
356	Image drift	Sample drift	We recommend checking and tuning the pressure and/or the valves of the optical table regularly. For visualizing the damping performance, we recommend the use of an accelerometer placed on the optical table and/or on the microscope vertical breadboard
		Objective drift	The drift correction option in the current analysis software will correct for the drift, provided that the samples are still in focus; however, fast drift in less than the time needed to acquire a single image stack file (usually of 3,000 frames, 30–60 s) will not be well corrected. Adding low-density beads to the samples is helpful for drift correction at the analysis stage. In addition, waiting ≥ 30 min after the sample is loaded before imaging might be helpful
		Temperature-related drifts	Make sure that the objective lock is operational, especially for long image acquisition Check and regulate temperature variations in the room

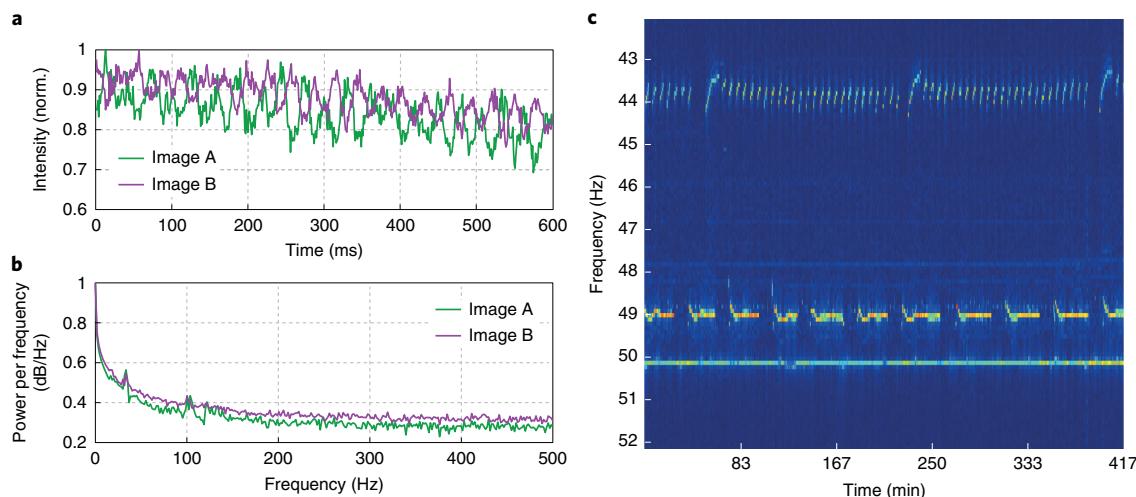


Fig. 23 | Example environmental vibration data. **a**, Anticorrelated intensity values of the same bead in different images indicate that vibrations lower the axial localization precision of the system. **b**, The power spectral density visualization of the same recording reveals pronounced frequencies. **c**, To find vibration sources nearby, it is helpful to plot recorded (audio) spectra as a function of time. Fingerprint-like patterns point to different sources, as seen for distinct patterns around 44 and 49 Hz in this case and 50 Hz (probably linked to the frequency of AC power delivery).

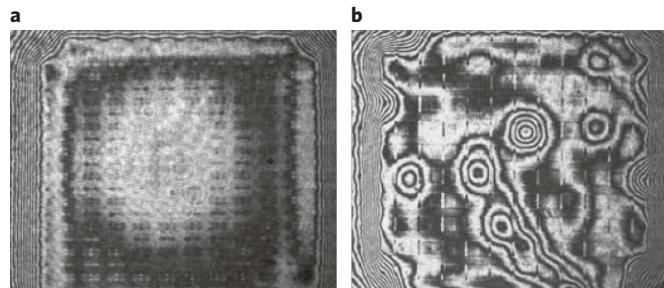


Fig. 24 | DM surface interference images. Interferograms for a functional (**a**) and damaged (**b**) DM.

Timing

As this is a complex microscope system, we give an approximation in the table below for the time that is required for key tasks during setup. The actual required time may vary substantially depending on the availability of parts and the experience and skill set of the personnel. Given the complexity of the system, ample time should be budgeted for troubleshooting and repetition of individual tasks that arise, for example, from correcting mistakes in machined parts or replacing broken components. With careful planning and focused work, a biophysicist or optical engineer, with help from biologists for specimen preparation, can set up the instrument and obtain images in 12 months.

Reagent setup, sample preparation: 1–2 d for biological samples (primarily because of incubation times), 1 h for bead samples

Equipment setup, room preparation: 1 week to 2 years in our experience, depending on the facility. This can vary widely and is highly dependent on the availability of suitable space. Space may already be available, or significant renovations may be required.

Equipment setup, lead times on components: Some key components such as paired objective lenses and DMs often have a long lead time of at least several months. These parts should be ordered early to avoid delays.

Equipment setup, software installation and familiarization: control software: 1 week; analysis software: 1 week

Steps 1–2, software installation and setup: 5–8 d

Steps 3–5, installation of optomechanical parts: 5–8 d

Steps 6–11, set up alignment laser and coupling: 4–8 h

Steps 12–68, emission beam path alignment: initial, 2–4 weeks; after becoming familiar, 2 d

Steps 69–101, excitation beam path alignment: 1–2 weeks

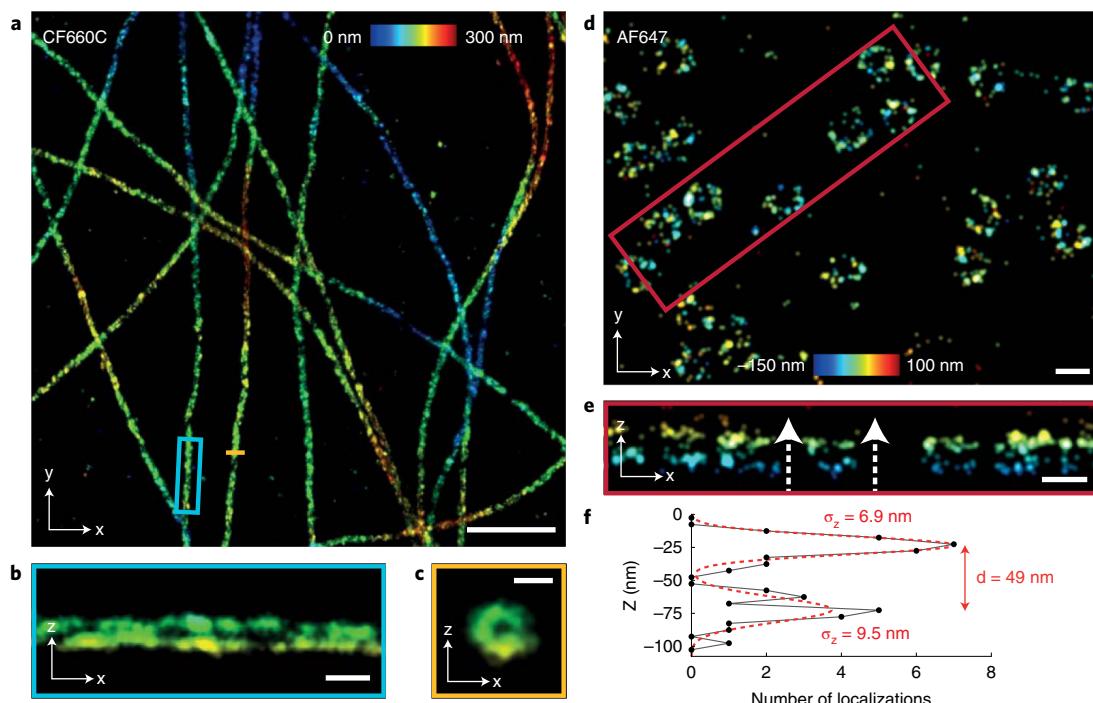


Fig. 25 | Example 4Pi-SMS images from immunostained microtubules and Nup96-SNAP labeled nuclear pore complexes. **a**, 4Pi-SMS image of indirectly immunostained α -tubulin labeled with CF660C in a COS7 cell in water-based imaging buffer. **b** and **c**, Axial views of the indicated regions in **a**. **d**, 4Pi-SMS image of Nup96-SNAP labeled with BG-Alexa 647 in the lower nuclear envelope of a U2OS cell in TDE-based index-matching imaging buffer. **e**, XZ projection of the indicated region in **d**. **f**, Fitting two Gaussians to the axial line profile of an individual nuclear pore complex quantifies the result. Scale bars are 1 μm (**a**), 100 nm (**b,d**, and **e**) and 50 nm (**c**).

Steps 102–141, imaging separation path alignment: 2–5 d
 Steps 142–153, sample mounting: 25–30 min
 Steps 154–186, loading a sample and setting OBJ1's lateral position: 60–90 min
 Steps 187–204, image setup and alignment: 2–4 h
 Steps 205–219, initial interference location: 1–5 d
 Steps 220–233, DM calibration and centering: 1–2 d
 Steps 234–248, phase adjustment across the field of view: 1–2 h
 Steps 249–273, quartz wedge calibration: 5–10 h
 Steps 274–294, DM system flat and astigmatism calibration: 30–60 min
 Steps 295–302, image registration: 1–5 min
 Steps 303–314, phase calibration: 5–20 min
 Steps 315–329, objective lock set up: 4–8 h
 Steps 330–342, mount a cell sample: 15–30 min
 Steps 343–347, image cell samples: 10–60 min
 Steps 348–356, data analysis: imaging data, 1–3 h per data set (single field of view, 40–120 cycles, computer dependent)

Anticipated results

It is advisable to test the imaging performance of the system using a test sample with well-known 3D structures (e.g., microtubules or nuclear pore complexes (Supplementary Methods)). The examples presented here are illustrations of the expected performance of the system when fully functioning.

Good cellular structures to test the performance of the system are microtubules, because they are sparsely dispersed, and the local density of labeling sites is high. Examples of expected images from microtubules in a COS7 cell labeled with CF660C are shown in Fig. 25a. If a sufficiently high 3D resolution is reached, the hollow structure of indirectly immunolabeled microtubules becomes clearly visible both in the side view (Fig. 25b) and in cross sections (Fig. 25c).

Another suitable test sample is the nucleoporin Nup96-SNAP²³, as its stereotypic structure serves as a resolution reference both laterally and axially. In the lateral view of the lower or upper nuclear

envelope, resolving individual corners of the nuclear pore complex proves an achieved lateral resolution better than 42 nm (Fig. 25d). In the axial direction, Nup96 forms two parallel rings 50 nm apart. As neighboring nuclear pore complexes typically have similar axial positions embedded in the nuclear envelope, two parallel lines become clearly visible when viewed from the side (Fig. 25e). Measuring the axial line profile for a single nuclear pore complex and fitting the sum of two Gaussians quantifies the structure (Fig. 25f). The distance d can be compared to the theoretical expectation of 50 nm and, hence, verifies the z-calibration. The standard deviations σ of the Gaussians indicate an upper bound of experimentally achieved axial localization precision.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Software and hardware plans are available from online repositories. The CAD file repository (Autodesk Inventor) can be found at <https://github.com/4Pi-SMS-consortium/CAD-files>. The complete parts list is available at <https://github.com/4Pi-SMS-consortium/CAD-files/blob/master/PartsList.xlsx> and as Supplementary Table 1. The microscope control software can be downloaded from <https://github.com/Gurdon-Super-Res-Lab/Microscope-Control>. The raw image files used to create Fig. 25a–c and Fig. 25d–f are available via the Zenodo online repositories: <https://zenodo.org/record/3929647#.X6Q-31NKgdU> and https://zenodo.org/record/4022827#.X6Q_GVNKgdU, respectively.

Code availability

The data analysis software referenced in this paper is available online at <https://github.com/4Pi-SMS-consortium/4Pi-SMS-analysis>.

References

1. Shtengel, G. et al. Interferometric fluorescent super-resolution microscopy resolves 3D cellular ultrastructure. *Proc. Natl Acad. Sci. USA* **106**, 3125–3130 (2009).
2. Aquino, D. et al. Two-color nanoscopy of three-dimensional volumes by 4Pi detection of stochastically switched fluorophores. *Nat. Methods* **8**, 353–359 (2011).
3. Wang, G., Hauver, J., Thomas, Z., Darst, S. A. & Pertsinidis, A. Single-molecule real-time 3D imaging of the transcription cycle by modulation interferometry. *Cell* **167**, 1839–1852.e21 (2016).
4. Huang, F. et al. Ultra-high resolution 3D imaging of whole cells. *Cell* **166**, 1028–1040 (2016).
5. Del Viso, F. et al. Congenital heart disease genetics uncovers context-dependent organization and function of nucleoporins at cilia. *Dev. Cell* **38**, 478–492 (2016).
6. Zhang, Y., Lara-Tejero, M., Bewersdorf, J. & Galan, J. E. Visualization and characterization of individual type III protein secretion machines in live bacteria. *Proc. Natl Acad. Sci. USA* **114**, 6098–6103 (2017).
7. Hwang, J. Y. et al. Dual sensing of physiologic pH and calcium by EFCAB9 regulates sperm motility. *Cell* **177**, 1480–1494.e19 (2019).
8. Schroeder, L. K. et al. Dynamic nanoscale morphology of the ER surveyed by STED microscopy. *J. Cell Biol* **218**, 83–96 (2019).
9. Zhang, Y. et al. Nanoscale subcellular architecture revealed by multicolor three-dimensional salvaged fluorescence imaging. *Nat. Methods* **17**, 225–231 (2020).
10. Karanastasis, A. A. et al. 3D mapping of nanoscale crosslink heterogeneities in microgels. *Mater. Horiz.* **5**, 1130–1136 (2018).
11. Brown, T. A. et al. Superresolution fluorescence imaging of mitochondrial nucleoids reveals their spatial range, limits, and membrane interaction. *Mol. Cell. Biol.* **31**, 4994–5010 (2011).
12. Schermelleh, L. et al. Super-resolution microscopy demystified. *Nat. Cell Biol.* **21**, 72–84 (2019).
13. Barentine, A. E. S. et al. 3D multicolor nanoscopy at 10,000 cells a day. Preprint at <https://www.biorxiv.org/content/10.1101/606954v1> (2019).
14. Booth, M. J. Adaptive optical microscopy: the ongoing quest for a perfect image. *Light Sci. Appl.* **3**, e165 (2014).
15. Burke, D., Patton, B., Huang, F., Bewersdorf, J. & Booth, M. J. Adaptive optics correction of specimen-induced aberrations in single-molecule switching microscopy. *Optica* **2**, 177–185 (2015).
16. Van Engelenburg, S. B. et al. Distribution of ESCRT machinery at HIV assembly sites reveals virus scaffolding of ESCRT subunits. *Science* **343**, 653–656 (2014).
17. Buttler, C. A. et al. Single molecule fate of HIV-1 envelope reveals late-stage viral lattice incorporation. *Nat. Commun.* **9**, 1861 (2018).
18. Liu, S. & Huang, F. Enhanced 4Pi single-molecule localization microscopy with coherent pupil based localization. *Commun. Biol.* **3**, 220 (2020).

19. Li, Y. et al. Accurate 4Pi single-molecule localization using an experimental PSF model. *Opt. Lett.* **45**, 3765–3768 (2020).
20. Li, X. et al. Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM. *Nat. Methods* **10**, 584–590 (2013).
21. Wang, Y. et al. Localization events-based sample drift correction for localization microscopy with redundant cross-correlation algorithm. *Opt. Express* **22**, 15982–15991 (2014).
22. Li, Y. et al. Real-time 3D single-molecule localization using experimental point spread functions. *Nat. Methods* **15**, 367–369 (2018).
23. Thevathasan, J. V. et al. Nuclear pores as versatile reference standards for quantitative superresolution microscopy. *Nat. Methods* **16**, 1045–1053 (2019).
24. Antonello, J., Wang, J., He, C., Phillips, M. & Booth, M. Interferometric calibration of a deformable mirror. Zenodo. 19 March 2020 (accessed 21 November 2020) <https://zenodo.org/record/3714951#.X6RM2lNKgdU>

Acknowledgements

We thank Fang Huang for advice, discussions and original code for the analysis software; Jacopo Antonello for advice, discussions and help with setting up the DMs; Chao He for assembling the DM calibration tool; David Miguel Susano Pinto for help and discussions with the analysis code; Andrew Barentine and Zach Marin for help and discussion about the analysis code; and David Baddeley for imaging advice, helpful discussions and help with the analysis code. J.W., M.A.P. and I.M.D. were supported by John Fell Fund award 141/144 and Wellcome Trust awards 105605/Z/14/Z and 107457/Z/15/Z. E.S.A. was supported by Wellcome Trust awards 095927/B/11/Z and 203285/Z/16/Z. G.S. was supported by Wellcome Trust awards 095927/B/11/Z and 203144/Z/16/Z. Y.Z., K.H., M.D.L. and J.B. were supported by Wellcome Trust awards 095927/A/11/Z and 203285/B/16/Z and National Institutes of Health (NIH) award R01 GM118486. K.H. was additionally supported by NIH award T32EB019941. R.D. was supported by an award from the Engelhorn Foundation. J.R. and Y.L. were supported by European Research Council award ERC CoG-724489, funding from the EMBL and the 4D Nucleome/4DN NIH Common Fund award U01 EB021223. Y.L. was additionally supported by the EMBL Interdisciplinary Postdoc Programme (EIPOD) under Marie Curie Actions COFUND and a start-up grant from the Southern University of Science and Technology, China. M.J.B. was supported by European Research Council award AdOMIS 695140 and Wellcome Trust award 203285/C/16/Z.

Author contributions

Hardware development: J.B., M.J.B., Y.Z., M.A.P., J.W., Y.L., E.S.A. and G.S.; software development: Y.Z., Y.L. and E.S.A.; specimen/imaging protocols: Y.Z. and M.D.L.; alignment protocols: E.S.A., G.S., J.W., Y.L., Y.Z. and K.H.; index matching protocol: R.D., J.R. and Y.L.; project supervision: J.B., M.J.B., J.R. and I.M.D.; writing and editing of the manuscript: all authors.

Competing interests

J.B. has financial interests in Bruker Corp. and Hamamatsu Photonics. J.B. is co-inventor of a US patent application (US20170251191A1) related to the 4Pi-SMS system and image analysis used in this work. Y.Z. and J.B. have filed a US patent application about the salvaged fluorescence multicolor imaging method described in this work.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41596-020-00428-7>.

Correspondence and requests for materials should be addressed to I.M.D. or J.R. or M.J.B. or J.B.

Peer review information *Nature Protocols* thanks Ilaria Testa and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 15 May 2020; Accepted: 25 September 2020;

Published online: 16 December 2020

Related links

Key references using this protocol

Huang, F. et al. *Cell* **166**, 1028–1040 (2016): <https://doi.org/10.1016/j.cell.2016.06.016>

Zhang, Y. et al. *Nat. Methods* **17**, 225–231 (2020): <https://doi.org/10.1038/s41592-019-0676-4>

Zhang, Y. et al. *Proc. Natl Acad. Sci. USA* **114**, 6098–6103 (2017): <https://doi.org/10.1073/pnas.1705823114>

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection Image data was collected with a custom program written in National Instruments (NI) LabVIEW 2016 64-bit, NI DAQmx, and NI Vision Development Module.

Data analysis Image data was analyzed with custom code written in MatLAB 2016 or later (64-bit) using resources from CUDA toolkit 10.2 (64-bit), and DIPIimage (64-bit). Vutara SRX 6.04.07 was used to visualize reconstructed 3D images. ImageJ 1.50c was used for line profile generation.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Software and hardware plans are available from the following online repositories: CAD File Repository (Autodesk Inventor): <https://github.com/4Pi-SMS-consortium/CAD-files>; Parts List: <https://github.com/4Pi-SMS-consortium/CAD-files/blob/master/PartsList.xlsx>; Hardware Control Software: <https://github.com/Gurdon-Super-Res-Lab/Microscope-Control>. Example Data (Figure 26a) is currently available via the following online repository: <https://drive.google.com/open?id=1I3vBu9UyKaMJ6h1eGCE8DSaslcNGrG9X>. The corresponding authors are available to address any technical questions upon reasonable request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

Life sciences Behavioural & social sciences Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	N/A
Data exclusions	N/A
Replication	N/A
Randomization	N/A
Blinding	N/A

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
<input type="checkbox"/>	<input checked="" type="checkbox"/> Antibodies
<input type="checkbox"/>	<input checked="" type="checkbox"/> Eukaryotic cell lines
<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology and archaeology
<input checked="" type="checkbox"/>	<input type="checkbox"/> Animals and other organisms
<input checked="" type="checkbox"/>	<input type="checkbox"/> Human research participants
<input checked="" type="checkbox"/>	<input type="checkbox"/> Clinical data
<input checked="" type="checkbox"/>	<input type="checkbox"/> Dual use research of concern

Methods

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input checked="" type="checkbox"/>	<input type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

Antibodies

Antibodies used	Mouse Anti- α -Tubulin (Sigma Aldrich, T5168)
Validation	Commercial antibodies were consistent with specifications provided by vendors, where over 2000 references are listed.

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)	COS7 ATCC batch 63624240
Authentication	Cell lines were purchased directly from ATCC and not independently authenticated.
Mycoplasma contamination	No contamination tests were carried out.
Commonly misidentified lines (See ICLAC register)	N/A