Constructing a cost-efficient, high-throughput and high-quality single-molecule localization microscope for super-resolution imaging

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Single-molecule localization microscopy (SMLM) leverages the power of modern optics to unleash ultra-precise structural nanoscopy of complex biological machines in their native environments as well as ultra-sensitive and high-throughput medical diagnostics with the sensitivity of a single molecule. To achieve this remarkable speed and resolution, SMLM setups are either built by research laboratories with strong expertise in optical engineering or commercially sold at a hefty price tag. The inaccessibility of SMLM to life scientists for technical or financial reasons is detrimental to the progress of biological and biomedical discoveries reliant on super-resolution imaging. In this work, we present the NanoPro, an economic, high-throughput, high-quality and easy-to-assemble SMLM for super-resolution imaging. We show that our instrument performs similarly to the most expensive, best-in-class commercial microscopes and rivals existing open-source microscopes at a lower price and construction complexity. To facilitate its wide adoption, we compiled a step-by-step protocol, accompanied by extensive illustrations, to aid inexperienced researchers in constructing the NanoPro as well as assessing its performance by imaging ground-truth samples as small as 20 nm. The detailed visual instructions make it possible for students with little expertise in microscopy engineering to construct, validate and use the NanoPro in <1 week, provided that all components are available.

Introduction

Many biological molecules assemble into nanoscopic complexes to perform their biological functions. Visualizing these tiny machines is of paramount importance to understanding the inner workings of the cell and, therefore, the human body in health and disease. Single-molecule localization microscopy (SMLM) is, undoubtedly, one of the most powerful super-resolution imaging tools developed over the last two decades to chart the nanoscopic organization of complex biological macromolecular assemblies in their native environments. Famous techniques, such as stochastic optical reconstruction microscopy (STORM), photo-activated localization microscopy (PAINT), and point accumulation in nanoscale tomography (PAINT), fall within the larger umbrella of SMLM. The general principle underlying the operation of this set of technologies is that, instead of imaging the bulk of fluorophores in a sample of interest, fluorophores are allowed to be switched 'on' and 'off' one at a time so that their centroids can be localized with high, single-digit nanometer precision. By repeating this process multiple times, the large majority of all fluorophores can be precisely located. These 'localizations' are then used to construct a super-resolved image that reveals the underlying biological structure with much higher clarity. The use of SMLM has uncovered the ultrastructure of many important biological macromolecular complexes including the nuclear pore complex, neuronal cytoskeleton, apoptotic pores, amyloid aggregates, endocytic machinery and many others.

Although the use and optimization of these different methods has been extensively pursued in the past, they all require individual fluorophores to be imaged with high precision and accuracy. High precision, resulting from the use of bright fluorophores, efficient detection schemes and precisely aligned illumination source(s), and high accuracy, resulting from simple, mechanically stable constructions that are robust against external vibrations and thermal drift, define in a complex manner the resolving power of a system. State-of-the-art microscopy setups delivering high resolving powers are available only commercially, are expensive to acquire and maintain (costing several...
hundreds of thousands of US dollars) and can be limited in their capabilities. Some of these microscopy setups are even delivered with additional features that are not required to perform super-resolution imaging. Among these features are eyepieces, which are no longer used to observe a mounted sample; objective and dichroic turrets, which are not required given the need for a single objective and dichroic mirror; and single-use control equipment (i.e., joystick), which delivers a suboptimal user experience. Furthermore, the large variety in microscopy components, as in lasers, cameras, stages and bodies, requires their integration by using generic microscopy control software solutions that can be tricky to use, specifically if high-throughput measurements are sought. Overall, these attributes render the use of SMLM in important biomedical investigations that require high-quality results in a high-throughput manner limited to laboratories and imaging facilities that have optical expertise or can afford tailored, but expensive, solutions.

Open-source microscopies and components for single-molecule imaging

The microscopy community has long advocated for the development of single-molecule microscopy setups that can be assembled from standard, off-the-shelf optical components, are substantially cheaper than commercially available, pre-assembled microscopy setups and are exclusively designed for SMLM experiments. To this end, four different microscopy setups were conceived in the last 5 years each addressing one or more challenges in the accessibility of single-molecule imaging by the wider scientific community. The first of these is the miCube (Fig. 1a), a modular fluorescence microscope developed by the Hohlbein laboratory that costs between US$23,000 (for the basic edition) and US$127,000 (for the premium edition, which includes a four-laser engine and high-end piezo stage). The miCube is constructed from a combination of simple 3D-printed parts and small computer numerically controlled (CNC) machined aluminum parts and includes an off-the-shelf, medium-to-high-sensitivity scientific complementary metal oxide semiconductor (sCMOS) camera,
an X/Y stage with a long (100 mm) travel range, a short-range piezo Z insert and a fiber-based laser combiner\textsuperscript{15}. The miCube can perform 3D single molecule imaging\textsuperscript{7,18} and single molecule FRET measurements\textsuperscript{19} and can be combined with a flat-field imaging module\textsuperscript{20}. The miCube was recently used in tracking the nuclease CRISPR–Cas9 with 40-nm precision in living, gram-positive bacteria. Despite its many features and remarkably small footprint, the miCube can be expensive if opting for the premium version; cannot be used for automated, multi-sample or long measurements given the lack of a focus stabilization system. The K2 total internal reflection (TIR) fluorescence (TIRF) microscope (https://ganzingerlab.github.io/K2TIRF/K2TIRF/index.html) is an alternative open-source microscope that was later developed by the Ganzinger laboratory with additional features to overcome several of the shortcomings of the miCube chassis (Fig. 1b), including the implementation of a focus stabilization system, flat-field illumination module and simultaneous multi-color imaging, but at the expense of a reduced capacity to perform multi-sample measurements because of the installation of a shorter-range X/Y stage, larger price tag (~US$137,000) due to the additional components and increased assembly complexity.

The second microscope setup is the liteTIRF (Fig. 1c) developed by the Jungmann laboratory for high-resolution PAINT imaging entirely assembled from off-the-shelf components at a moderate cost of US$24,000 (single edition)\textsuperscript{21}. To lower its cost, the setup uses one single-wavelength laser, an economic sCMOS camera with moderate quantum efficiency, a manual X/Y/Z stage and a highly compacted assembly that uses a minimum number of components. liteTIRF was shown to resolve DNA origami structures with 10-nm spaced docking strands. Although the demonstrated resolution (i.e., localization precision and accuracy) is remarkable, this was achieved through iterative alignment of the imaged origamis, taking advantage of prior knowledge of their structure. Despite achieving a single-digit nanometer resolution, the setup cannot be used for multi-colored single-molecule experiments because of the presence of single-laser, high-throughput, multi-sample imaging due to the absence of a motorized X/Y/Z stage, intensity-sensitive measurements due to the absence of a flat-field illumination module and general-purpose single-molecule imaging experiments due to the use of a low-quality camera that is not sensitive enough for capturing the low number of photons released by single fluorophores under different imaging modalities. Unlike the miCube and the K2 TIRF, the liteTIRF is not a modular assembly because of its highly compacted structure. This implies that expansion of the setup to include more laser lines or a larger, higher-quality camera might not be possible without changing the assembly architecture.

The third open-source microscope setup is the WOSM (Warwick open-source microscope; Fig. 1d), an ultra-stable CNC machined assembly developed by the Cross laboratory for high-quality super-resolution imaging (https://wosmic.org/). The WOSM chassis is a sturdy, two-piece, low-profile aluminum assembly that largely resembles the miCube. An important selling point of the microscope is the minimized distance between the mounted sample and the top side of the chassis, which, according to its developers, allows for long-duration, high-resolution imaging without the need for drift correction. Notably too, and similar to the K2 TIRF, the WOSM comes with a comprehensive control suite. Despite its numerous advantages, particularly its remarkable stability, the WOSM is, presumably, substantially more expensive than the other microscopes, given the use of the highest-quality components (e.g., a high-sensitivity electron multiplying charge coupled detector camera and commercial laser combiner). It is also not suited for high-throughput, multi-sample imaging because of short travel range on the X/Y/Z stage and lack of a focus stabilization system, is not capable of performing intensity-sensitive measurements due to the absence of a flat-field illumination module and can be complicated to construct, given the need for long milling components required to drill through a large, single block of aluminum forming the base of the chassis. Although the development of WOSM can be traced back as far as 2016, the setup is not yet presented with a complete list of components required for its assembly.

These revolutionary microscopes are 1.5 to 20 times cheaper than commercially available microscopes, which can range in price from US$170,000 (for separate commercial components) to US $400,000 (for bespoke, pre-assembled solutions). Although the reduction in price is remarkable, other developers focused their efforts on developing substantially cheaper instruments, but at the expense of reduced imaging quality. The first of these microscopes was developed by the Liu laboratory and is an open-frame setup that costs ~US$3,800 (Fig. 1e)\textsuperscript{22}. The setup is composed of ultra-low-cost and off-the-shelf components that would allow imaging in an epi-illumination mode and is, therefore, best suited for STORM imaging only. Although robust quantitative resolution measurements are lacking, results based on imaging microtubules and the H3 protein demonstrate a spatial resolution of
30–100 nm. The setup cannot be used for high-speed, high-quality PAINT or, more generally, TIRF imaging because of limitations on the illumination angle (which cannot be controlled on the setup, because the excitation beam cannot be translated, and because the setup uses an optical diffuser that homogenizes the beam but creates several secondary point sources), is not suited for long-duration (>10,000 frames) imaging because of unestablished stability measurements, cannot be used for high-throughput, multi-sample imaging because of the absence of a motorized X/Y/Z stage and requires a custom-written imaging script. The second, and final, of the cost-efficient setups is cellSTORM2 (https://beniroquai.github.io/stormocheap/), a 3D-printed, mobile phone–based super-resolution microscope costing <US$1,000 (Fig. 1) that is an upgrade to the previously developed cellSTORM23. cellSTORM2 can perform super-resolution imaging with 100-nm spatial resolution. To reduce the cost of cellSTORM2, the developers used a combination of 3D-printed and ultra-low-cost optomechanical components, a waveguide chip and deep learning for image processing. Although this is clearly remarkable, the notable reduction in price comes at the expense of a significantly reduced spatial resolution and inability to perform multi-sample imaging experiments.

These microscopes and others24 have pioneered the broad accessibility of SMLM but collectively suffer from three important shortcomings that prevent them from achieving their vision. The first shortcoming is that none of the above microscopes (except for cellSTORM and to some extent miCube) is provided with a step-by-step guide for assembly. These microscopes were designed with the vision of being accessible to scientists with little optical expertise, who have tight budgets and who need a super-resolution microscope to answer an important biological or biomedical question. To meet this vision, it is absolutely essential that the end user is capable of assembling and maintaining these relatively sophisticated machines with ease. The second shortcoming is that most of the listed setups are not designed for high-throughput, multi-sample imaging. Biological and biomedical scientists can draw meaningful scientific conclusions only by imaging several cellular and human samples under various conditions. For this reason, and because these assemblies are designed for biologists, high-throughput, multi-sample imaging counts as a fundamentally important feature to have. The third, and final, shortcoming is that none of the above microscopes is delivered with a simple user control experience. Being versatile, gaming controllers are being extensively used across many modern instruments, including commercial microscopes such as Nanoimager from Oxford Nanoimaging and OpenStage25, to deliver an engaging user experience. The use of simple control tools is seen as important for the accessibility of these instruments by the wider community of non-experts.

The above microscopes are standalone devices that have undergone extensive optimization in design to serve specific purposes. Some developers adopted a different direction, which is to optimize the individual components that comprise any microscope assembly. As an example, the Ries laboratory has recently developed a highly economic laser combiner (https://github.com/ries-lab/LaserEngine), taking advantage of cheap, high-power diode lasers26. The downside of using such lasers is having to cope with their distorted beam profile, which strongly deviates from the Gaussian shape commonly encountered with high-quality, but expensive, lasers. To overcome this problem, they combine the different lasers into a single, square-core, multi-mode fiber to produce a square profile, flat-field illumination that can be used for high-quality single-molecule experiments. This trick can reduce the price of a laser combiner from US$45,000 to <US$5,000. Another example is the open flexure stage (https://openflexure.org/), which attempts to democratize high-quality microscopes through the development of high-precision, sub-micron-resolution stages out of 3D-printed components25. Although, to our knowledge, these stages were tested only with low-to-medium-quality super-resolution imaging26, they hold much promise because of their versatility and the strong support provided for their production, assembly and use. A final example is NanoJ (https://github.com/HenriquesLab/NanoJ-Fluidics/wiki) developed by the Henriques laboratory, which relies on the use of LEGO blocks to construct an open-source fluidic exchange system that can be easily integrated with any open-source or commercial microscope system25. These, and more, components, or rather subassemblies, can be used in many different ways to construct robust, multi-modal microscopes at a fraction of the price.

Overview of NanoPro 1.0
Extending the revolutionary work described above, and mitigating its major shortcomings, as well as building on our expertise in constructing high-throughput, multi-sample SMLM setups, we designed and conceived the NanoPro 1.0, an economic, ultra-high-quality custom microscope
solution for high-throughput, multi-sample super-resolution imaging based on the detection of single molecules (Fig. 2a and Tables 1 and 2). NanoPro 1.0 is assembled from a combination of CNC machined aluminum parts and off-the-shelf components to produce a high-line super-resolution microscope for <US$70,000 (2.4–5.7 times cheaper than existing commercial rivals). The microscope is specifically intended for biological and biomedical scientists with no prior expertise in optical engineering who require an autonomous machine for high-throughput, multi-sample and multi-target super-resolution imaging at 20-nm spatial resolution and that can be easily assembled by using the detailed, IKEA style, step-by-step instructions provided in this protocol. This microscope is not intended for individuals or facilities that require ultra-low-cost microscopes, setups with ultra-high-stability and spatial resolutions much below 20 nm, setups with minimal features but high-to-modest spatial resolutions and microscopes with an extensive set of features but increased assembly complexity. The many excellent alternative open-source microscopes described in the previous section would better serve these diverse purposes. NanoPro 1.0 achieves its unique positioning in the cost-versus-quality space by implementing the following features:

1. An economic, multi-laser engine powered by high-power, low-cost lasers coupled into a custom-made square-core fiber at remarkable coupling efficiencies (>80%) to deliver square profile, flat-field illumination at the sample plane. The square-core has a 70 µm × 70 µm core that is smaller than commercially available fibers used in contemporary setups with low-cost laser/light-emitting...
| Attribute                                      | miCube | K2TIRF | liteTIRF | WOSM | Unnamed low-cost microscope | cellSTORM | ONI Nanomage | NanoPro 1.0 |
|------------------------------------------------|--------|--------|----------|------|----------------------------|------------|--------------|-------------|
| Demonstrated spatial resolution (nm)           | <50    | Unknown| <10 (with origami-based drift correction) | Unknown | -110 (with computational drift correction) | -100 (without drift correction) | <20 (listed) (with computational drift correction) | <20 (with computational drift correction) |
| Temporal resolution (lowest exposure time at full frame) (ms) | 10 | 50 | 25 | 50 | 20 | 30 (depending on mobile phone used) | 10 | 10 |
| Active drift stabilization                     | No     | Yes    | No       | No   | No                         | No         | Yes          | Yes          |
| Flat-field illumination                        | Yes    | No     | No       | Medium | No                         | Yes        | Yes          | Yes          |
| Construction complexity                        | Low (partially illustrated) | High | Medium | Medium | Low (fully illustrated) | Pre-assembled | Yes          | Yes          |
| Multi-sample imaging                          | Yes, but not drift stable | Yes, medium range (30-mm X/Y stage movement) | No | No | No | Yes, medium range (30-mm X/Y stage movement) | Yes, long range (50-mm X/Y stage movement) |
| Multi-color imaging                            | Yes    | Yes    | No       | No   | No                         | No         | Yes          | Yes          |
| 3D imaging                                     | Yes    | Yes    | No       | No   | No                         | No         | Yes          | Yes          |
| Analysis and post-processing                  | No     | No     | No       | No   | No                         | No         | Yes          | Yes          |
| Illumination modes                             | Epi HILO TIRF | Epi HILO TIRF | Epi HILO TIRF | Epi HILO TIRF | Epi HILO | Epi HILO TIRF | Yes        | No |
| Temperature control                            | No     | Yes    | No       | No   | No                         | Yes        | Yes          | Yes          |
| Environment requirements                      | None listed | None listed | None listed | None listed | None listed | None listed | None listed | None         |
| Setup cost for full features (not including maintenance or service) (US$) | ~127,000 | ~137,000 | 24,000 | ~70,000-125,000 | ~3,800 | 1,000 | N/A | 68,000 |

Data from refs. 16,21–23. N/A, not available.
| Cost category               | Cost for year 1 (US$) | Cost for year 2 (US$) | Cost for year 3 (US$) | Cost for year 4 (US$) | Cost for year 5 (US$) |
|----------------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| Direct costs—equipment     |                       |                       |                       |                       |                       |
| Fiber (items 1 and 2)      | 1,400                 | 0                     | 0                     | 0                     | 0                     |
| Light (items 3–9)          | 7,600                 | 0                     | 0                     | 0                     | 0                     |
| Light analysis (items 10–14) | 13,800                | 0                     | 0                     | 0                     | 0                     |
| Motion control (items 15–18) | 10,400                | 0                     | 0                     | 0                     | 0                     |
| Optics (items 19–40)       | 16,300                | 0                     | 0                     | 0                     | 0                     |
| Optoelectronics (items 41–62) | 4,300                 | 100 (vibration motor replacement) | 100 (vibration motor replacement) | 100 (vibration motor replacement) | 100 (vibration motor replacement) |
| Optomechanics (items 63–134) | 13,700                | 0                     | 0                     | 0                     | 0                     |
| Sample (items 125–136)     | 2,900                 | 0                     | 0                     | 0                     | 0                     |
| Safety (item 137)          | 200                   | 0                     | 0                     | 0                     | 0                     |
| Software (items 138–139)   | 0                     | 0                     | 0                     | 0                     | 0                     |
| Other (items 140–144)      | 200                   | 0                     | 0                     | 0                     | 0                     |
| **Total direct costs**     | **70,800**            | **1,100**             | **2,100**             | **1,100**             | **2,100**             |
| Indirect costs—personnel   |                       |                       |                       |                       |                       |
| Stage 1 (procurement and space preparation) (6 months at 0.05% FTE of one PhD student) | 2,200 | 0 | 0 | 0 | 0 |
| Stage 2 (fabrication)      |                       | 0 (included in direct costs) | 0                     | 0                     | 0                     |
| Stage 3 (unpacking and installation) (2 d at 100% FTE of four PhD students) | 1,900 | 0 | 0 | 0 | 0 |
| Stage 4 (assembling the laser combiner) (3 h at 100% FTE of one PhD student) | 30 | 0 | 0 | 0 | 0 |
| Stage 5 (first partial assembly of the microscope box) (1 h at 100% FTE of one PhD student) | 10 | 0 | 0 | 0 | 0 |
| Stage 6 (assembling the excitation module and focus-stabilization system) (2 h at 100% FTE of one PhD student) | 20 | 0 | 0 | 0 | 0 |
| Stage 7 (second partial assembly of the microscope box (1 h at 100% FTE of one PhD student) | 10 | 0 | 0 | 0 | 0 |
| Stage 8 (assembling the emission module) (1 h at 100% FTE of one PhD student) | 10 | 0 | 0 | 0 | 0 |
| Stage 9 (assembling the sample stage) (2 h at 100% FTE of one PhD student) | 20 | 0 | 0 | 0 | 0 |
| **Total indirect costs**   | **25,500**            | **100**               | **200**               | **100**               | **200**               |
Table 2 (continued)

| Cost category                                                                 | Cost for year 1 (US$) | Cost for year 2 (US$) | Cost for year 3 (US$) | Cost for year 4 (US$) | Cost for year 5 (US$) |
|-------------------------------------------------------------------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| Stage 10 (setting up electrical connections) (2 h at 100% FTE of one PhD student) | 5                     | 0                     | 0                     | 0                     | 0                     |
| Stage 11 (assembling the laser-combiner enclosure) (0.5 h at 100% FTE of one PhD student) | 20                    | 0                     | 0                     | 0                     | 0                     |
| Stage 12 (assembling the sample enclosure) (2 h at 100% FTE of one PhD student) | 5                     | 0                     | 0                     | 0                     | 0                     |
| Stage 13 (assembling and aligning the sample holder) (0.5 h at 100% FTE of one PhD student) | 20                    | 0                     | 0                     | 0                     | 0                     |
| Stage 14 (setting up the computer and installing the NanoPro software) (2 h at 100% FTE of one PhD student) | 40                    | 0                     | 0                     | 0                     | 0                     |
| Stage 15 (aligning the laser combiner) (4 h at 100% FTE of one PhD student) | 5                     | 0                     | 0                     | 0                     | 0                     |
| Stage 16 (aligning the excitation and emission paths) (0.5 h at 100% FTE of one PhD student) | 5                     | 0                     | 0                     | 0                     | 0                     |
| Stage 17 (aligning the focus-stabilization system) (0.5 h at 100% FTE of one PhD student) | 5                     | 0                     | 0                     | 0                     | 0                     |
| Stage 18 (measuring the camera pixel size) (0.5 h at 100% FTE of one PhD student) | 5                     | 0                     | 0                     | 0                     | 0                     |
| Stage 19 (imaging ground-truth samples to establish performance) (1 h at 100% FTE of one PhD student) | 10                    | 0                     | 0                     | 0                     | 0                     |
| Stage 20 (data processing) (1 h at 100% FTE of one PhD student) | 10                    | 0                     | 0                     | 0                     | 0                     |
| Maintenance and troubleshooting (50 h at 100% FTE of one PhD student) | 0                     | 500                   | 500                   | 500                   | 500                   |

| Total indirect costs | - | 4,300 | 500 | 500 | 500 | 500 |
| Total (direct and indirect) costs | - | 75,100 | 1,600 | 2,600 | 1,600 | 2,600 |

Direct and indirect costs are shown as well as projected maintenance costs up to 5 years. Full-time equivalent (FTE) for PhD students is calculated at the highest published salary worldwide (https://ethz.ch/en/the-eth-zurich/working-teaching-and-research/welcome-center/employment-contract-and-salary/salary.html), which is 80,520 Swiss francs (equivalent to US$87,000). FTE for technicians is calculated at 1.5 times the FTE for PhD students, which amounts to US$130,500. Figures above US$100 are approximated to the nearest US$100. Inflation and the time value of money are not included.
diode (LED) combiners\textsuperscript{26,34}. Because of its small core size, our fiber system can be used to illuminate a sample of interest in epifluorescence, highly inclined laminated optical sheet (HILO) or TIR modes to allow high-quality STORM, PAINT or generic single-molecule-based experiments (Fig. 2b and Supplementary Fig. 2). The laser engine is housed in an economic, easy-to-assemble and stable enclosure to comply with laser safety regulations.

A focus-stabilisation system assembled from widely available optomechanical components and controlled by using the custom software provided with NanoPro 1.0 and the multipurpose controller delivered with the assembly (Fig. 2c). The focus-stabilisation system facilitates prolonged imaging by adjusting for axial drift and is fundamentally important for the automation of multi-sample imaging (Supplementary Fig. 1).

A slip-stick X/Y/Z stage with 50-mm travel range and 1-nm movement resolution in all directions (Fig. 2d). The stage differentiates itself from traditional motorized X/Y stages and piezo-based Z stages in that it allows long range travel in the lateral directions, which, when coupled with the focus-stabilization system described above, permits high-throughput, multi-sample imaging. These features come in an economic assembly that is at least half the price of competing alternatives.

Multi-color imaging enabled by a four-slot, low-cost and off-the-shelf filter changer (Fig. 2e). Although lacking in all other open-source microscopies, this important feature would allow hybrid, FRET-based techniques (e.g., FRET-PAINT\textsuperscript{35}) to be easily conceived, which allows conventional PAINT experiments to be accelerated by up to 100 fold.

Best-in-class sCMOS camera with 95% maximum quantum efficiency, 6.5-µm pixel size and low electron readout noise at a third of the price of conventional electron multiplying charge-coupled device (EMCCD) cameras (Fig. 2f). The camera’s high quantum efficiency, homogeneous noise profile and small-to-moderate pixel size allow signal oversampling. This results in high-quality single-molecule imaging with median localization precisions reaching down to 2.1 nm, only 1 nm more than sophisticated and complex microscopy configurations such as MINFLUX\textsuperscript{36}.

A transparent enclosure that protects the microscope, minimizes disturbance to the mounted sample from air currents and permits the controlled flow of gases to cater for the future possibility of performing live-cell super-resolution imaging (not demonstrated here; Fig. 2g).

A game pad controller that controls the lasers, focus-stabilization system, stage, filter changer, camera’s live mode and acquisitions (Fig. 2h). The controller elevates the pressure of the graphical user interface and delivers an engaging experience that is intuitive and economic.

A fully illustrated, step-by-step guide (i.e., this protocol) that explains, with ample detail, the assembly, operation, performance evaluation and troubleshooting of NanoPro 1.0, assuming no prior expertise in optical engineering or knowledge of optical, and related, components.

Although this protocol focuses on the use of NanoPro 1.0 in super-resolution imaging, because of the lack of ground-truth, commercially available reference standards for single-molecule tracking, distancing and counting experiments, it is expected that these measurements would be readily achievable on our setup, given the presence of all the required quality components.

### Choice of components

In order for us to guarantee the high quality of our instrument, as well as the presence of the necessary features mentioned above, we focused on acquiring components that can perform the needed functions reliably, can be easily and reproducibly integrated into the entire assembly, are economic and can be purchased from reliable vendors with worldwide shipping services or be manufactured at local facilities. There is usually a strong competition between products that occupy a unique niche in the market, such as scientific-grade cameras and positioners (i.e., motorized sample stage), in which case the choice between these different products was dictated by quality over price. Expensive, high-quality products with additional, unrequired features were replaced with the next best competitor product on the market if it did not compromise the purpose of our assembly. In addition, preference was given to products that are more expensive but can be reliably and reproducibly integrated within the entire assembly to ensure that the end user can easily replicate this protocol.

As a first example of the above, the choice of excitation filters (i.e., dichroic filters used to combine the different laser lines) was based on the cost and ease of installation in widely available optomechanical components. However, this was not the case with filters and dichroic mirrors in the emission path, which were chosen for the highest quality to ensure that imaging quality, particularly the localization precision, is not compromised.
As a second example, the choice had to be made between expensive (>US$500), high-quality, low-power lasers; low-cost (US$100–500), low-quality, high-power lasers; and ultra-low-cost (<US$100), ultra-low-quality, high-power lasers. As previously mentioned, beam shape quality does not correlate with imaging quality in our setup, because all the combined laser lines are launched into a square-core fiber that produces homogeneous illumination at the sample plane. This leaves us with the low- and ultra-low-cost lasers to choose from. Ultra-low-cost lasers, also known as laser diodes, suffer from several important shortcomings. Among these are undocumented lifetime measurements; lack of quality control over the output intensity; imprecise engineering, resulting in unexpected designs and tolerances; the need for packaging by the end user; and the need for the laser temperatures to be controlled by using external, complicated electronic circuitry. Low-cost lasers do not suffer from these shortcomings. Given that the price difference between a low-cost and an ultra-cost laser would not exceed US$400, which in the grand scheme of the entire assembly represents <2% of the total cost, choosing a more expensive, but still low-cost, laser that can be easily integrated and operated is sensible.

A third example is that of the cameras, which are available in three categories: expensive, ultra-low-noise and low-speed EMCCD cameras, which, for more than a decade, were the camera of choice for single-molecule imaging experiments; low-cost, low-noise and high-speed sCMOS cameras; and ultra-low-cost, medium-noise and high-speed non-scientific CMOS cameras. NanoPro 1.0 was designed to provide the highest-quality single-molecule imaging (i.e., highest localization precision and highest signal-to-noise ratio). Compromising imaging quality with the use of a non-scientific CMOS camera was out of the question, and, therefore, the choice was left between the EMCCD and sCMOS cameras. sCMOS cameras are being increasingly used for super-resolution imaging because of their superiority in detecting single molecules at high photon counts. Until recently, they were manufactured with peak quantum efficiencies of 82%. Currently available, equally-priced sCMOS cameras are produced with a 95% peak quantum efficiency and intelligent noise-correction algorithms, which can push the localization precision of single molecules detected in PAINT and STORM experiments down to 2 nm, as well as, allow faithful single-molecule tracking, distancing and counting experiments in which the photon budget is low. Furthermore, currently available sCMOS cameras can be run at 5 to 10 times higher speeds than the best-in-class EMCCD cameras at full field of view, provide stable operation at higher temperatures, are manufactured with up to four times the number of pixels, are gaining a larger share in the global market for cameras and are up to a third cheaper in price than EMCCDs. All these attributes were strong indicators for us to consider the use of an sCMOS camera in our instrument.

The fourth and final example is that of the X/Y/Z stage. Conventional, commercially available microscopes are fitted with a motorized X/Y stage and Z stage composed of a motorized part for coarse movement and a piezo-positioner for fine focusing and movement. Although this configuration offers a long travel range along the X and Y axes, which is suitable for imaging samples mounted on glass coverslips and multi-well plates, single-molecule and, particularly, super-resolution experiments are performed on glass coverslips on which no more than 50 mm of travel range is required, and, therefore, the extra-long travel range is not required for these measurements. In addition, this system is expensive, costing ≥US$23,000, and is relatively troublesome to control because of the presence of multiple different components that use different coding libraries. There are two alternatives to this configuration: a low-cost, open-loop, piezo-electric 3-axis actuator or a moderate-cost, closed-loop, piezo-electric 3-axis actuator, both of which can be readily configured to move along the X, Y and Z axes. The open-loop option does not contain a position sensor. This would prevent the end user from returning to a recorded or absolute position, given the high error in movement reproducibility. Although an open-loop configuration can be used with random multi-field-of-view imaging, it would not be suitable for imaging at periodic or user-recorded positions. It is often the case that the end user desires to image at set locations, in which case the open-loop system would not serve their purpose. To this end, we chose the slightly more expensive closed-loop positioners to ensure that multi-sample imaging is not restricted as described above.

The above examples are not inclusive of all the components used in the microscope assembly. The choice of these components was dictated by the set of rules, rationally designed for constructing the NanoPro 1.0, which were described earlier. These rules ensure that the microscope is well suited for the highest-precision structural measurements. At times, this comes at a cost. Users who can sacrifice precision for a substantially lower cost, may consider replacing some of the components listed in the procedure below with those listed in Table 3.
Key performance indicators

Establishing the performance of a super-resolution microscope is an important task that helps the end user answer a basic, but important, question: what is the smallest distance between any two biological objects/structures that a microscope can resolve? Answering this question requires the use of a ground-truth sample, one in which the dimensions of the underlying structure(s) are known a priori. There are numerous ground-truth samples that can be used to measure the spatial resolution of a microscope. To choose the best in class and ensure that our results are easily reproducible by the end user, our sample had to satisfy the following requirements:

1. It must be purchased ready for mounting. This excludes a large number of excellent samples including immuno-stained nuclear pore complexes, actin filaments, microtubules and clathrin-coated pits, which have to either be obtained from collaborators with strong and established expertise in producing these samples to ensure high-quality control standards in their production or be prepared in-house, in which case quality cannot be appropriately controlled.

2. It can be ordered in a range of sizes, below and above the predicted spatial resolution of the system. Many of the biological samples listed above are larger than the predicted spatial resolution of NanoPro 1.0 (i.e., 20 nm). As an example, the average diameter of the nucleoporins forming nuclear pore complexes lies between 70 and 120 nm\(^{37}\), and the average diameter of microtubules is ~100 nm\(^{38}\). Although image-based algorithms, such as the Fourier Ring Correlation\(^{39}\), can be used to quantify the spatial resolution of a system from images acquired from these samples, seeing is believing. The end user has the right to not just quantify the spatial resolution of the system but be able to confirm that they can observe biological objects at that limit.

3. It can be imaged with different dyes. As previously mentioned, the spatial resolution of a system is dependent on the localization precision, which in turn is dependent on the quality of detection at the emission wavelength of the fluorophore used in an experiment. This might be a deterrent to using some of the cellular samples listed above in some imaging modalities, such as DNA-PAINT, in which some dyes can be seen to bind to cellular structures in a nonspecific manner.

4. It can be ordered with fiducial markers. Nearly every microscope requires correction against thermal and mechanical drift through the application of drift-correction algorithms, based on cross-correlation\(^{40}\), or by using fiducial markers, such as fluorescent beads or nanoparticles, or a combination of these two approaches. The spatial resolution of a microscope system is clearly dependent on which of these methods is used for drift correction. As such, the sample to be used for establishing the performance of NanoPro 1.0 has to contain fiducial markers to allow the end user to quantify the lower limit of the spatial resolution.

DNA origami structures are the only ground-truth samples that satisfy the above requirements. A DNA origami is a precisely engineered array of different, single-stranded DNA molecules held together by a DNA scaffold\(^{41,42}\). These tiny structures were originally developed for a range of applications in nanotechnology but found their way in developing DNA-PAINT\(^{7}\) and their subsequent use in establishing the resolving power of PAINT-based microscopes. The preparation of DNA origami structures is facilitated by Picasso\(^{11}\), a software tool that can be readily used to design and simulate origami structures in which docking strands can be placed along a 2D periodic array at distances as short as 2.5 nm and up to 50 nm. The complication in using origami structures is that their assembly is a meticulous process requiring the careful addition of >150 different DNA strands at fixed proportions. Imperfections in the synthesized strands or a single error during the assembly process can result in faulty structures that are not suitable for establishing the performance of a microscope.

To ameliorate this problem, we reverted to commercially available origami structures, known as ‘nanorulers’, which are composed of three equidistant docking strands placed in a line at set dimensions of 20 and 40 nm. These nanorulers are pre-assembled and pre-mounted on sealed coverslips that are delivered ready to use. To this end, we used the NanoPro 1.0 in imaging the nanorulers as described in the protocol (see Procedure). Using a simple cross-correlation drift-correction algorithm, we could readily uncover the ultra-structure of the 40- and 20-nm nanorulers (Fig. 3a,b). Our drift-correction curves (Fig. 3c) demonstrate that the NanoPro 1.0 is exceptionally stable, is capable of excluding frame-to-frame perturbations and allows image recovery without the use of fiducial markers or sophisticated drift-correction algorithms for structures as small as 20 nm. Furthermore, our median localization precision (2.1 nm; Fig. 3d) measurements indicate that our instrument can rival the best-in-class, commercially available systems and can provide localization precisions that are only 1.1 nm higher than those provided with alternative, non-single-molecule-based methods such as MINFLUX.
The stability and precision of the NanoPro 1.0 are strong indicators of its suitability for high-quality super-resolution imaging.

Usage and limitations

As with any microscope setup, NanoPro 1.0 comes with a number of limitations that inform its usage. To aid the end user in choosing whether to construct the instrument described here, or else how to best use it, we have decided to divide these limitations into five mutually exclusive categories: sample-, readout-, accuracy-, design- and investment-based limitations.

With regards to sample-based limitations, the NanoPro 1.0 was designed for high-throughput, multi-sample super-resolution imaging. As such, the sample holder designed for our instrument can accommodate the following: 26 × 76 mm, #1.5 thickness glass coverslips (VWR, cat. no. MENZB-C026076AC40), alone or coupled with CultureWell gaskets composed of fifty 3-mm wells (Grace Bio-Labs, cat. no. GBL103250), as well as Ibidi µ-Slide 8- or 18-well glass-bottom chambers. These hosts...

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**Table 3 | Alternative components, their pricing and their effect on localization precision**

| Component (item no.) | Alternative component | Vendor (cat. no.) | Price difference (US$) | Effect on localization precision |
|-----------------------|-----------------------|-------------------|------------------------|---------------------------------|
| Apochromatic, 100× magnification, 1.49 numerical aperture, TIRF objective (item 19) | 100× magnification, 1.3 numerical aperture objective | Thorlabs (N100X-PFO) | 5,600 | 0.5 nm (theoretical calculations); effect on TIRF imaging unquantified; effect on multi-color imaging unquantified |
| Prime BSI Express, sCMOS camera (item 14) | Chameleon3 CM3-U3-31S4M, PointGrey CMOS camera | Edmund Optics (36-075) | 11,000 | 0.5–1 nm (theoretical calculations); effect on multi-color imaging unquantified |

Data from ref. 22.

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**Fig. 3 | Assessing the performance of NanoPro 1.0 on ground-truth nanoruler samples.**

(a, b) Drift-corrected DNA-PAINT images of 40-nm (a; scale bar = 10 μm (100 nm for inset)) and 20-nm (b; scale bar = 10 μm (100 nm for inset)) nanorulers (see Materials). (c) Exemplary drift-correction curves as produced by a cross-correlation algorithm (X shown in green and Y shown in blue). (d) Localization precision histogram under typical imaging conditions (see Procedure). Counts (×10,000).
can accommodate up to 50 different recombinant, cellular or human-derived samples at volumes ranging from 200 to 3 µl per sample, to be imaged in a single run. Furthermore, and given that these silicon gaskets can be readily mounted, the coverslips can be functionalized in various ways to enable not only super-resolution imaging but also surface-based, single molecule–based assaying.

As for readout-based limitations, the microscope is not capable of performing (i) measurements of single-molecule FRET because of the absence of wavelength-splitting equipment in the emission path, (ii) 3D SMLM imaging because of the absence of a cylindrical lens or other optical elements for engineering the point spread function or (iii) spectrally resolved SMLM because of the absence of a diffraction grating or dispersive prism in the emission path. Although some of these methods are already well established, the decision not to integrate them was based on the rationale of simplifying both the assembly and readout to what is commonly required. Nevertheless, given the modular nature of the instrument, some or all of these features may be included in future upgrades.

With regard to accuracy-based limitations, the NanoPro 1.0 achieves 20-nm spatial resolution by using simple algorithms based on cross-correlation for drift correction. A higher resolution might be achieved by using iterative alignment of origami-based fiducial markers. However, deep axial imaging might prevent the user from appropriately observing these markers, and some surface-preparation protocols could be incompatible with their incorporation. Other drift-correlation algorithms, such as those based on residual entropy, were reported to achieve single-digit nanometer resolution; however, these were not tested here, and, therefore, their performance cannot be guaranteed.

As for the design-based limitations, although the NanoPro 1.0 is more compact than many of the commercially available setups, it is assembled on a 1.5 m × 0.9 m optical table and, therefore, requires at least 2.5 m × 1.5 m of room space. The instrument is also not suitable for placement inside an enclosed biosafety cabinet.

Finally, even though the NanoPro 1.0 is an economic, state-of-the-art setup providing many of the advanced features delivered with commercial microscopes at a fraction of the price, it still requires a substantial financial investment as well as partially dedicated research staff to build and maintain the instrument as described here (Table 2).

These five important factors, when considered with the set of biological or biomedical questions that need to be answered, should allow the end user to inform a balanced decision on whether it is appropriate to establish our instrument in their own laboratory or facility and how to best make use of it.

**Overview of the procedures**

The protocol starts by planning for the procurement, fabrication and installation of all components making up the NanoPro 1.0 as well as obtaining access, and negotiating for, a suitable room to accommodate the microscope’s assembly. We first describe how to assemble the setup, starting with the laser engine, microscope box, focus-stabilization system, detection module, sample stage and holder and, finally, all enclosures. We then describe how to set up the electrical connections and install the NanoPro 1.0 control software to align the lasers and focus-stabilization system before assessing the performance of the system and operating it thereafter. Due to the sheer number of components comprising, and steps involved in building, the assembly, we have introduced the following:

1 The assembly, alignment and operation procedures are accompanied by detailed, illustrated guides (the visual assembly, alignment and operation guides; see Code availability) that assume no expertise in mechanical or optical engineering.

2 The reference number of each item (see Materials) is listed once in each section to ensure that similar items are not confused.

**Materials**

**Equipment**

**Fiber**

- (Item 1) 2× 70 µm × 70 µm, numerical aperture (NA) = 0.22, length (L) = 2 m, FC/PC connector, square-core fiber cable (CeramOptec, cat. no. 05806-1 Rev. A)
- (Item 2) 40-mm focal length, FC/PC connector, achromatic fiber collimator (Thorlabs, cat. no. C40FC-A)

**Light**

- (Item 3) 1× 561-nm, 100-mW laser (Roithner LaserTechnik, cat. no. RLTMLL-561-100-3)
- (Item 4) (optional) 1× 10-KHz analog modulation unit (Roithner LaserTechnik, cat. no. RLMX1 ANALOG 10KHZ)
• (Item 5) 1× 350-mW, 405-nm diode laser with cooling option (Lasertack, cat. no. PD-01254-E)
• (Item 6) 1× 55-mW, 488-nm diode laser with cooling option (Lasertack, cat. no. PD-01339-E)
• (Item 7) 1× 700-mW, 635-nm diode laser with cooling option (Lasertack, cat. no. PD-01229-E)
• (Item 8) 1× 20-mW, 850-nm compact diode laser module with shutter (Thorlabs, cat. no. LDM850)
• (Item 9) 1× 740-mW, 4,900-K, 1,225-mA mounted LED (Thorlabs, cat. no. MNWHL4)

Light analysis
• (Item 10) 1× 320–1,100-nm 2D lateral effect position sensor (Thorlabs, cat. no. PDP90A)
• (Item 11) 1× 700–1,400-nm IR detector card (Thorlabs, cat. no. VRC5)
• (Item 12) 1× Digital liquid crystal display (LCD), compact power and energy meter console (Thorlabs, cat. no. PM100D)
• (Item 13) 1× 400–1,100-nm, 500-mW standard photodiode power sensor (Thorlabs, cat. no. S121C)
• (Item 14) 1× Prime BSI Express sCMOS camera (Teledyne Photometrics, cat. no. O1_PRIME_BSI_EXP)

Motion control
• (Item 15) 3× enhanced blocking force and integrated sensor, 51-mm travel positioner (Smaract, cat. no. SLC-1780-D-S)
• (Item 16) 1× MSC2 sensor module (Smaract, cat. no. MCS2-S-0001)
• (Item 17) 1× MSC2 control system (Smaract, cat. no. MCS2-C-0002)
• (Item 18) 1× K-Cube photo-sensitive detector (PSD) auto aligner (Thorlabs, cat. no. KPA101)

Optics
• (Item 19) 1× apochromatic, 100× magnification, 1.49-numerical aperture TIRF objective (Nikon, cat. no. MRD01991)
• (Item 20) 1× 405/488/561/635/800–1,050-nm BrightLine multiphoton super-resolution dichroic splitter (Semrock, cat. no. DI01-R405/488/561/635/800-T1-25X36)
• (Item 21) 1× 390/482/564/640-nm BrightLine quad-band bandpass filter (Semrock, cat. no. FF01-390/482/564/640-25)
• (Item 22) 1× 446/523/600/777-nm BrightLine quad-band bandpass filter (Semrock, cat. no. FF01-446/523/600/777-25)
• (Item 23) 1× 525/30-nm BrightLine single-band bandpass filter (Semrock, cat. no. FF01-525/30-25)
• (Item 24) 1× 600/37-nm BrightLine single-band bandpass filter (Semrock, cat. no. FF01-600/37-25)
• (Item 25) 1× 676/29-nm BrightLine single-band bandpass filter (Semrock, cat. no. FF01-676/29-25)
• (Item 26) 1× 700-nm BrightLine multiphoton short-pass dichroic beam splitter (Semrock, cat. no. FF700-SB01-25X36)
• (Item 27) 1× 425-nm, long-pass dichroic mirror (Thorlabs, cat. no. DMLP425)
• (Item 28) 1× 505-nm, long-pass dichroic mirror (Thorlabs, cat. no. DMLP505)
• (Item 29) 1× 605-nm, long-pass dichroic mirror (Thorlabs, cat. no. DMLP605)
• (Item 30) 1× 805-nm, long-pass dichroic mirror (Thorlabs, cat. no. DMLP805)
• (Item 31) 1× 700-nm, premium long-pass filter (Thorlabs, cat. no. FELH0700)
• (Item 32) 10× 400–750-nm, broadband dielectric mirror (Thorlabs, cat. no. BB1-E02)
• (Item 33) 1× 750–1,100-nm, broadband dielectric mirror (Thorlabs, cat. no. BB1-E03)
• (Item 34) 1× 125-mm focal length, achromatic doublet (Thorlabs, cat. no. AC254-125-A-ML)
• (Item 35) 1× 200-mm focal-length tube lens (Thorlabs, cat. no. TTL200-A)
• (Item 36) 1× 11-mm focal-length, 0.25-numerical aperture, aspheric lens (Thorlabs, cat. no. C220TMD-A)
• (Item 37) 1× 1.0 optical density, neutral density filter (Thorlabs, cat. no. NE10B-A)
• (Item 38) 1× 700–1,100-nm, 30-mm, non-polarizing beam splitter (Thorlabs, cat. no. CCM1-BS014/M)
• (Item 39) 1× variable line-grating test target (Thorlabs, cat. no. R1L3S6P)
• (Item 40) 1× pack of one thousand 26 × 76 mm, #1.5 Menzel Glaser glass coverslips (VWR, cat. no. MENZBC026076AC40)

Optoelectronics
• (Item 41) 1× Intel Core i9 processor, NVIDIA GeForce GTX 1650 Ti 4 GB, 32 GB RAM, 1 TB solid-state drive laptop (Dell, XPS 15 (9500))
• (Item 42) 1× 7-port universal serial bus (USB) desktop hub (Currys, cat. no. ACH115EU)
(Item 43) 1× Sony PlayStation DualShock 4 controller (Onecall, cat. no. CS30605)
(Item 44) 1× 4-output digital analog converter device (Active Robots, cat. no. 1002_0B)
(Item 45) 1× 6-output control hub (Active Robots, cat. no. HUB0000_0)
(Item 46) 1× 5-V constant DC power source device (Active Robots, cat. no. PSU2000_0)
(Item 47) 1× 1.5–5-V variable DC power source device (Active Robots, cat. no. PSU2001_0)
(Item 48) 2× 60-cm mini-USB cable (Active Robots, cat. no. 3036_0)
(Item 49) 2× 60-cm converter device cable (Active Robots, cat. no. 3002_0)
(Item 50) 1× 12-outlet surge-protected power strip (Thorlabs, cat. nos. HDPS12-UK (for the United Kingdom), HDPS12-US (for the United States) and HDPS12-EU (for the European Union))
(Item 51) 1× 1-m black cable trunking (Thorlabs, cat. no. CMS002)
(Item 52) 1× 15-V, 2.4-A power supply unit for one K- or T-cube (Thorlabs, cat. no. KPS101)
(Item 53) 1× 15-V/5-V power supply unit for K- or T-cubes (Thorlabs, cat. no. TPS002)
(Item 54) 1× T-cube LED driver (Thorlabs, cat. no. LEDD1B)
(Item 55) 2× 914-mm, male-to-male Bayonet Neill-Concelman (BNC) coaxial cable (Thorlabs, cat. no. 2249-C-36)
(Item 56) 2× BNC to test clips (Thorlabs, cat. no. T3788)
(Item 57) 3× 12-V, 80-W, 100–240-V AC power supply for laser modules (Lasertack, cat. no. PD-01341-E)
(Item 58) 1× 30-A, 250-V terminal strip (RS Components, cat. no. 782-2857)
(Item 59) 1× 10-m unscreened flat ribbon cable (RS Components, cat. no. 214-0683)
(Item 60) 3× 1-m International Electrotechnical Commission (IEC) power cable (RS Components, cat. nos. 731-6185 (for the United Kingdom), 731-6163 (for the United States) and 626-6751 (for the European Union))
(Item 61) 5× 5-mm vibration motor (Precision Microdrives, cat. no. 304-111)
(Item 62) 1× 50-cm Micro B to USB A 2.0 cable (XMA, cat. no. CDL-160-05M)

Optomechanics

(Item 63) 1× 700 mm × 900 mm × 1,500 mm heavy-duty passive frame (Thorlabs, cat. no. PFH90150-8)
(Item 64) 1× 110 mm × 900 mm × 1,500 mm breadboard (Thorlabs, cat. no. B90150B)
(Item 65) 1× passive-isolation foot pump (Thorlabs, cat. no. PTA127)
(Item 66) 1× fiber launch system (Thorlabs, cat. no. KT110/M)
(Item 67) 1× four-position slider bundle (Thorlabs, cat. no. ELL9K)
(Item 68) 4× 225-mm square construction rail (Thorlabs, cat. no. XE25L225/M)
(Item 69) 12× 50-mm-length, 25-mm-diameter pedestal pillar post (Thorlabs, cat. no. RS2P/M)
(Item 70) 4× 38-mm-length, 25-mm-diameter pedestal pillar post (Thorlabs, cat. no. RS1.5P/M)
(Item 71) 1× 2-inch-length, 1-inch-diameter pedestal pillar post (Thorlabs, cat. no. RS2P)
(Item 72) 2× pack of five 50-mm-length, 12.7-mm-diameter optical posts (Thorlabs, cat. no. TR50/M-P5)
(Item 73) 1× pack of five 30-mm-length, 12.7-mm-diameter optical posts (Thorlabs, cat. no. TR30/M-P5)
(Item 74) 3× pack of five 50-mm-length, 12.7-mm-diameter post holders (Thorlabs, cat. no. PH50/M-P5)
(Item 75) 1× 1.24-inch-slot-length clamping fork (Thorlabs, cat. no. CF125)
(Item 76) 9× pack of four 0.5-inch-length, 6-mm-diameter cage assembly rods (Thorlabs, cat. no. ER05-P4)
(Item 77) 8× 0.25-inch-length, 6-mm-diameter cage assembly rod (Thorlabs, cat. no. ER025)
(Item 78) 2× pack of four 6-mm diameter rod adapters (Thorlabs, cat. no. ERSCB-P4)
(Item 79) 4× kinematic mirror mount (Thorlabs, cat. no. KM100CP/M)
(Item 80) 3× 30-mm right-angle kinematic mirror mount (Thorlabs, cat. no. KC81C/M)
(Item 81) 3× 30-mm cage cube with dichroic filter mount (Thorlabs, cat. no. CM1-DCH/M)
(Item 82) 3× 30-mm, 0.5-inch-thick cage plate (Thorlabs, cat. no. CP33T/M)
(Item 83) 8× compact kinematic mirror mount (Thorlabs, cat. no. KMS/M)
(Item 84) 8× 1-inch-diameter, 2.5–6.1-mm-thick mirror holder (Thorlabs, cat. no. MH25)
(Item 85) 3× rotation mount for 1-inch optics (Thorlabs, cat. no. LRM1)
(Item 86) 1× 2-inch-travel SM1 zoom housing (Thorlabs, cat. no. SM1NR1)
(Item 87) 3× SM1 end cap (Thorlabs, cat. no. SM1CP2)
(Item 88) 2× SM1 coupler (Thorlabs, cat. no. SM1T2)
(Item 89) 2× adapter with external SM1 threads and internal SM2 threads (Thorlabs, cat. no. SM1A2)
(Item 90) 1× adapter with external SM1 threads and internal M25 × 0.75 threads (Thorlabs, cat. no. SM1A12)
• (Item 91) 1× adapter with external SM05 threads and internal SM1 threads (Thorlabs, cat. no. SM1A1)
• (Item 92) 1× adapter with external C-mount threads and external SM1 threads (Thorlabs, cat. no. SM1A39)
• (Item 93) 1× 2-inch SM1 lens tube (Thorlabs, cat. no. SM1L40)
• (Item 94) 1× pack of five 0.3-inch SM1 lens tubes (Thorlabs, cat. no. SM1L03-P5)
• (Item 95) 1× 0.5-inch SM1 lens tube without external threads (Thorlabs, cat. no. SM1M05)
• (Item 96) 1× 1-inch SM1 lens tube spacer (Thorlabs, cat. no. SM1S10)
• (Item 97) 1× FC/PC fiber adapter cap with internal SM1 threads (Thorlabs, cat. no. S120-FC)
• (Item 98) 1× SM1 thread spanner wrench (Thorlabs, cat. no. SWP606)
• (Item 99) 1× nine-piece hex key set (Thorlabs, cat. no. CCHK/M)
• (Item 100) 1× 4-40 cap screw and hardware kit (Thorlabs, cat. no. HW-KIT5)
• (Item 101) 1× M6 cap screw and hardware kit (Thorlabs, cat. no. HW-KIT2/M)
• (Item 102) 1× pack of twenty-five 0.25-inch-20 thread, 0.5-inch-length stainless steel setscrew (Thorlabs, cat. no. SS25S050)
• (Item 103) 1× pack of two microscope slide spring clips (Thorlabs, cat. no. SLH1/M)
• (Item 104) 1× bullseye level (Thorlabs, cat. no. LVL01)
• (Item 105) 1× pack of two handles (Thorlabs, cat. no. BBH1)
• (Item 106) 2× hinge for rail enclosures (Thorlabs, cat. no. XE25H)
• (Item 107) 1× lid stop for rail enclosures (Thorlabs, cat. no. XE25LS)
• (Item 108) (optional: consult workshop) 2× 20 mm × 235 mm × 275 mm 5080 aluminum tool plate (Aalco, cat. no. 277120)
• (Item 109) (optional: consult workshop) 2× 20 mm × 160 mm × 235 mm 5080 aluminum tool plate (Aalco, cat. no. 277121)
• (Item 110) (optional: consult workshop) 2× 20 mm × 200 mm × 275 mm 5080 aluminum tool plate (Aalco, cat. no. 277122)
• (Item 111) (optional: consult workshop) 3× 20 mm × 105 mm × 130 mm 5080 aluminum tool plate (Aalco, cat. no. 277124)
• (Item 112) (optional: consult workshop) 1× 20 mm × 105 mm × 230 mm 5080 aluminum tool plate (Aalco, cat. no. 277125)
• (Item 113) (optional: consult workshop) 1× 20 mm × 80 mm × 90 mm 5080 aluminum tool plate (Aalco, cat. no. 277129)
• (Item 114) (optional: consult workshop) 1× 20 mm × 45 mm × 105 mm 5080 aluminum tool plate (Aalco, cat. no. 277131)
• (Item 115) 1× wire stripper (RS Components, cat. no. 663-617)
• (Item 116) 1× pack of fifteen 32-mm-length, 6-mm-diameter plain steel dowel pins (RS Components, cat. no. 270-653)
• (Item 117) 1× pack of twenty 24-mm-length, 4-mm-diameter plain steel dowel pins (RS Components, cat. no. 270-596)
• (Item 118) 1× pack of one hundred 20-mm-length M1.6 TORX stainless steel screws (RS Components, cat. no. 179-5712)
• (Item 119) 1× pack of ten 15 mm × 15 mm steel angle brackets (RS Components, cat. no. 427-991)
• (Item 120) 1× pack of two hundred and fifty 0.3-mm-thick M1.6 stainless steel plain washer (RS Components, cat. no. 179-5724)
• (Item 121) 1× pack of 10 M6 clamping knobs (RS Components, cat. no. 830-4158)
• (Item 122) 1× planar connecting element (Smaract, cat. no. SCE-CN)
• (Item 123) 1× rectangular connecting element (Smaract, cat. no. SCE-RN)
• (Item 124) 1× TORX T5 screwdriver (Farnell, cat. no. 4432850)
• (Item 125) 1× 2.5 mm × 50 mm slotted screwdriver (Farnell, cat. no. 3378478)
• (Item 126) 1× 525-piece M3 to M6 socket cap screw kit (Onecall, cat. no. TRFAKIT0005)
• (Item 127) (optional: consult workshop) 1× 2,440 mm × 1,220 mm × 5 mm black foam polyvinyl chloride (PVC) (Vision Plastics)
• (Item 128) (optional: consult workshop) 1× 1,000 mm × 1,000 mm × 8 mm clear Perspex (Engineering & Design Plastics)
• (Item 129) 1× laser plates (custom fabrication)
• (Item 130) 1× laser combiner enclosure (custom fabrication)
• (Item 131) 1× positioner base (custom fabrication)
• (Item 132) 1× microscope box (custom fabrication)
• (Item 133) 1× sample enclosure (custom fabrication)
• (Item 134) 1× sample holder (custom fabrication)

Sample
• (Item 135) 1× 40-nm Cy3B immobilized high-resolution DNA-PAINT nanorulers (GATTAcquant, cat. no. 3030)
• (Item 136) 1× 20-nm Cy3B immobilized high-resolution DNA-PAINT nanorulers (GATTAcquant, cat. no. 3031)

Safety
• (Item 137) 1× 12% visible-light-transmission, universal-style laser safety glasses (Thorlabs, cat. no. LG4)

Software
• (Item 138) Software to control NanoPro 1.0 is available at https://github.com/jdanial/NanoPro.
• (Item 139) ImageJ software (https://imagej.nih.gov/ij/)

Other
• (Item 140) 1× low-autofluorescence immersion oil (Thorlabs, cat. no. MOIL-30)
• (Item 141) 1× fiber and vibration motor mount (custom fabrication)
• (Item 142) 1× lens-cleaning tissue (Merck, cat. no. WHA2105862)
• (Item 143) 1× pack of 25 thick Viton O-rings (Hooper, cat. no. OR26X2V175)
• (Item 144) 1× pack of 50 thin Viton O-rings (Hooper, cat. no. OR26X1V175)
• (Item 145) 1× 25.1-mm-focal-length Plano convex lens (Thorlabs, cat. no. LA1951-ML)
• (Item 146) 1× 1-inch SM1 lens tube (Thorlabs, cat. no. SM11L10)

Procedure

Stage 1: procurement and space preparation  ● Timing ≤6 months
1  To guarantee the high performance (i.e., maximized localization accuracy and precision) of NanoPro 1.0, obtain access to a suitable room that satisfies the following requirements:
• Is on the ground, or preferably basement, floor of the building and does not face, or is not close to, a street. Having the setup on the ground or basement floors avoids low-frequency vibrations that cannot be dampened by using the air isolation table (item 63) and that can affect the resolving power of the microscope. The host institution can be consulted on whether the building can accommodate such an optical table on a basement or ground floor room.
• Is laminated with a vibration-isolation material and, if possible, is mechanically isolated from the rest of the building.
• Is fitted with card access or a keypad lock as well as a signal device to indicate when the microscope is under operation (i.e., when lasers are on).
• Is fitted with an air conditioner for controlling the temperature and humidity.
• Is ≥4.5 m × 3.5 m in dimensions. The door of the room should be ≥85 cm wide, and the hallway along which the room is located should be ≥1.5 m wide. All hallways and doors leading to the room should be ≥1.35 m wide.
• Does not contain any large equipment that generates heat or noise (e.g., freezers, pumps and robots).
• Does not contain any windows or other uncontrollable light sources. This room could contain windows if they are blacked out.
2  Procure (i.e., order) all components one after the other in the following order: positioners and all auxiliary components (items 15, 16, 17, 122 and 123), objective lens (item 19), lasers (items 3, 4, 5, 6, 7 and 57), fiber cable (item 1), filters (items 20–26) and samples (items 135 and 136), followed by all other items. The following should be considered when items are procured:
• For the positioners and all their auxiliary components, the supplier should be asked for all the necessary screws; this is a collection of M2 and M1.6 screws that are only produced by the supplier and that are required for assembly.
• For the lasers, the supplier should be requested not to fix the adjustable lenses by using glue or any other material and to ensure that the rotating threads are extended out of the laser heads for fine adjustment of collimation.
For the optical table (items 63 and 64), the supplier should be, first, advised with a suitable date for delivery. These are large and heavy items, and they need to be dismantled (i.e., unpackaged; see Stage 3: unpacking and installation) and delivered inside the building as fast as possible to prevent road blockages. Second, the supplier should be requested to place the two items comprising the optical table far from one another to ensure easy unpacking. Third, the supplier should be requested to provide on-site unpackaging and installation of the optical table. If agreed, Steps 9–17 can be skipped.

- All suppliers should be asked for delivery notes to satisfy grant requirements (if applicable).
- Some of the components are listed with UK-based suppliers. The catalog number of these components is shared between worldwide suppliers and can be ordered by using these numbers from local suppliers.

3 Manufacture items 129–134 in a mechanical workshop where quality can be easily monitored and controlled (e.g., a mechanical workshop facility within the institute or department). Because of the precision required in fabricating these components, it is advised that these components are not procured from companies that perform accelerated, computerized manufacturing and prototyping because of the high cost incurred for the accelerated service and the lack of control of quality. Although complete instructions on assembly are provided below, it is advised that the mechanical workshop assembles these components in their ready-to-use form to ensure reproducibility during assembly (for further information, see Stage 2: fabrication).

Stage 2: fabrication ● Timing ≤3 months

4 Follow these instructions for manufacturing the laser plates (item 129):
- If applicable, provide the mechanical workshop with the aluminum tool plates (items 111 and 112).
- Provide the mechanical workshop with drawing files (Drawing 1.pdf and Drawing 2.pdf) and CAD files (CAD_File 1.f3d and CAD_File 2.f3d) (see Code availability).

5 Follow these instructions for manufacturing the microscope box, positioner base and sample holder (items 131–133):
- If applicable, provide the mechanical workshop with the aluminum tool plates (items 108, 109, 110, 113 and 114).
- Provide the mechanical workshop with drawing files (Drawing 3.pdf—Sheets 1–9) and CAD files (CAD_File 3 to 10.f3d) (see Code availability).

6 Follow these instructions for manufacturing the sample enclosure (item 134):
- If applicable, provide the mechanical workshop with the clear Perspex (item 128).
- Provide the mechanical workshop with drawing files (Drawing 4.pdf) and CAD files (CAD_File 11 to 17.f3d) (see Code availability).

7 Follow these instructions for manufacturing the laser combiner enclosure and fiber and vibration motor mount (items 130 and 141):
- If applicable, provide the mechanical workshop with the black foam PVC (item 127).
- Provide the mechanical workshop with drawing files (Drawing 5.pdf and Drawing 6.pdf) and CAD files (CAD_File 20 to 26.f3d) (see Code availability) for the laser combiner enclosure.
- Provide the mechanical workshop with the supplementary CAD file (CAD_File 18.f3d) for 3D printing the fiber and vibration motor mount (see Code availability).

Stage 3: unpacking and installation ● Timing 2 d

8 Clear the microscope room of all equipment and packages that were delivered beforehand to ensure that there is enough space for installing the optical table (items 63 and 64).

▲ CRITICAL STEP At least eight individuals need to carry out Steps 9–17. These individuals need to be contacted well ahead to ensure their presence on the day of delivering the optical table. Furthermore, ensure that none of the contacted individuals is medically unfit for lifting heavy material (25 kg).

▲ CRITICAL STEP Steps 9–17 involve the unpackaging and lifting of heavy material. Lifting heavy material is a serious risk. To avoid this risk, the supplier can be contacted well ahead to arrange an on-site unpackaging and installation service, as advised in Step 2. In case the supplier agrees, Steps 9–17 can be skipped. In case the supplier does not agree, a risk assessment form (see risk assessment form and guidance (Form 1.pdf) in Code availability) needs to be filled out to ensure that all activities are compliant with health and safety regulations.
CRITICAL STEP  Request from the goods-in or mechanical workshop facilities a set of large hex keys to unpack the optical table. Obtaining a cordless screwdriver set is recommended where available.

CRITICAL STEP  Request from the goods-in or mechanical workshop facilities a forklift (also known as a hand pallet truck) to be used for lifting one of the two items comprising the optical table.

The optical table will be delivered in two large wooden pallets secured by long, Philips-head screws. For the larger pallet (i.e., the frame), loosen all the screws on the top and front/back wooden panels by using the screwdriver set borrowed from your local facility. Carefully remove the panels and stow them in a safe area.

CAUTION  A small number of the screws will be hard to remove. Note the location of these screws and avoid getting in contact with these, to prevent injury.

CAUTION  Wear thick cotton gloves and enclosed shoes before dismantling.

Pierce the plastic film wrapping the frame; with the aid of all participating individuals, lift the frame a few centimeters off the bottom of the wooden pallet, carefully slide it out of the wooden pallet, and, finally, rest it on the ground.

Lift the frame off the ground. Two individuals should lift the left, shorter side of the frame, and another two should lift the right, shorter side of the frame. Two of the four individuals should walk backward facing and the other two forward facing.

Deliver the frame to the microscope room and orient the frame so that the back, longer side is 30 cm away from a wall with at least one AC power socket protected by a circuit breaker to avoid any short circuits.

For the smaller pallet (i.e., the breadboard), loosen all the screws on the top, front, back, left and right wooden panels by using the screwdriver set borrowed from your local facility. Carefully remove the panels and stow them in a safe area.

Observe the gaps between the bottom of the breadboard and the wooden pallet. Insert the forklift (i.e., hand pallet truck) in the gaps. Push the handle of the forklift downward to lift the forks and the breadboard upward. The longer side of the breadboard will be perpendicular to the fork. To ensure better stability during delivery and allow easier access through narrow doors and hallways, carefully rotate the breadboard on the fork. One of the eight individuals has to hold the forklift in place to ensure that the breadboard does not slip off the fork. Drive the fork lift to the entrance of the microscope room, ensuring that someone holds the doors open.

Push the breadboard to slip it off the fork and allow its longer edge to touch the ground. Lift the breadboard from the other longer edge upward until the breadboard sits perpendicular to the ground.

Slide the breadboard (item 64) into the microscope room parallel to the installed frame (item 63) but ≥2 m away. Two of the eight individuals should tilt the breadboard such that it is almost level with the ground, but ensuring that a small gap remains along the long edge. At this point, two other individuals should lift the breadboard from each corner of the other long edge touching the ground.

Lift the breadboard and walk sideways to bring it on top of the installed frame. Carefully bring the level breadboard down and closer to the top of the frame, taking care not to trap hands and fingers between the breadboard and the frame. Rest the breadboard on the frame.

The four passive isolators (https://www.thorlabs.com/thorproduct.cfm?partnumber=PWA075) pre-installed on the frame, and on which the breadboard rests, have to be inflated. To inflate the isolators, bring the foot pump (item 65) close to one of the isolators. Loosen the black cap on the needle of the intended isolator. Press the clip on the hose of the foot pump and insert the hose into the needle (as shown in figure 1.3 of the following manual. https://www.thorlabs.com/drawings/6313cb5cb5f59e-BB0977FE-B35F-CCA5-D87443D134DE216/PWA075-Manual.pdf). Inflate the isolator by pressing on the foot pump until it is correctly inflated (as shown in figure 1.4 of the manual.). Press the clip and quickly release the hose off the needle. Screw the black cap on the needle.

Repeat Step 18 with the remaining three passive isolators.

Stow or discard all panels comprising the wooden pallets in which the optical table was delivered, as advised by the building administrator.

CAUTION  Unremoved screws or sharp objects protruding off the wooden pallet should be removed or bent inward to prevent serious injury.

Unpack the remaining components delivered beforehand and place them in a large space (e.g., floor of the microscope room) in numerical order (see Materials).
Stage 4: assembling the laser combiner  

**CRITICAL** The individual performing the assembly process should stand facing the longer edge of the breadboard (item 64). Wherever there is reference to the front side, this will be the long side closest to the individual performing the assembly process; wherever there is reference to the back side, this will be the long side furthest from the individual performing the assembly process.

**CRITICAL** For all items that need to be screwed to the breadboard, the screwing position is indicated by a two-coordinate label (X,Y), where X refers to the position along the longer side of the breadboard and Y refers to the position along the shorter side of the breadboard. The position (1,1) is the thread to the front/right hand side. Moving to the left increases the X position. Moving to the back increases the Y position.

22 Screw an M6 × 20 mm set screw (item 101) to the lower side of the 50-mm-long, 25-mm-wide pedestal pillar post (item 69) (Fig. 1 in the visual assembly guide; see Code availability). Repeat the same for the remaining 11 pedestal pillar posts.

23 Screw the (12) assemblies formed in the previous point to the breadboard at positions (7,3), (7,6), (11,3), (11,6), (7,13), (7,16), (11,13), (11,16), (7,18), (7,21), (11,18) and (11,21) (Fig. 2 in the visual assembly guide).

24 Repeat Step 22 with the four 38-mm-long, 25-mm-wide pedestal pillar posts (item 70) and screw them to the breadboard at positions (3,8), (3,11), (11,8) and (11,11) (Fig. 3 in the visual assembly guide).

25 Take one of the three small laser plates (item 129) and place it over the closest set of four 50-mm-long, 25-mm-wide, breadboard-screwed pedestal pillar posts from the front side. Screw the laser plate to the posts by using four M6 × 12 mm cap screws (item 101). Tighten the screws by using the 5-mm L-shaped hex key with the green band (item 99) (Fig. 4 in the visual assembly guide). Repeat the same for the remaining two small laser plates, screwing them to the second and third closest sets of four 50-mm-long, 25-mm-wide, breadboard-screwed pedestal pillar posts from the front side (Fig. 5 in the visual assembly guide).

26 Take the large laser plate (item 129) and place it over the closest set of four 38-mm-long, 25-mm-wide, breadboard-screwed pedestal pillar posts from the front side. Screw the laser plate to the posts by using four M6 × 16 mm cap screws (item 101). Tighten the screws by using the 5-mm L-shaped hex key with the green band.

27 The ultraviolet (405 nm), blue (488 nm) and red (638 nm) lasers (items 5, 6 and 7, respectively) are packaged in two separate components: a laser head and an electronic circuit board. Connect the male green terminal wired to one of the laser heads to the female green terminal, labeled LD+, LD−, NTC, NTC, TEC+ and TEC−, on the accompanying electronic circuit board (Fig. 6 in the visual assembly guide). Repeat the same for the remaining two lasers.

**CRITICAL STEP** Do not randomly connect any laser head with any electronic circuit board. The circuit boards are configured to work with the accompanying lasers. Connect laser heads to electronic circuit boards only from the same package.

28 Slot the board-connected ultraviolet (405 nm) laser head into the central groove of the furthest small laser plate from the front side. Screw the laser head to the laser plate by using two M5 × 12 mm cap screws (item 126). Tighten the screws by using the 4-mm L-shaped hex key with the cyan band (item 99) (Fig. 7 in the visual assembly guide). Place the connected electronic circuit board on top of the four 3-mm threads on the laser plate next to the recently fixed laser head. Secure the electronic circuit board by using four M3 × 12 mm cap screws (item 126). Fix the screws by using the 2-mm L-shaped hex key with the orange band (item 99) (Fig. 8 in the visual assembly guide). Repeat the same with the blue (488 nm) and red (638 nm) lasers, placing them on the second closest and closest small laser plates from the front side, respectively.

29 The green (561 nm) laser (items 3 and 4) is packaged in two separate components: a large laser head and a large control box. Slot the laser head into the central groove of the large laser plate. Secure the laser head by using four M4 × 25 mm cap screws (item 126). Tighten the screws by using the 3-mm L-shaped hex key with the blue band (item 99) (Fig. 9 in the visual assembly guide). Note that the laser head will be connected to the control box in Step 172.

30 Slot an M6 × 16 mm cap screw into a 50-mm post holder (item 74) (Fig. 10 in the visual assembly guide). Screw and tighten the cap screw into the thread on the post holder by using the 5-mm L-shaped hex key with the green band (Fig. 11 in the visual assembly guide). Repeat the same for another seven 50-mm post holders. Screw the eight post holders onto the breadboard at positions (16,4), (16,9), (16,14), (16,19), (14,7), (14,12), (14,17) and (14,22) (Fig. 12 in the visual assembly guide).
31 Screw a 50-mm-long, 12.7-mm-wide optical post (item 72) to a compact kinematic mirror mount (item 83) (Fig. 13 in the visual assembly guide). Repeat the same for the remaining seven 50-mm-long, 12.7-mm-wide optical posts and compact kinematic mirror mounts.

32 Dismantle the mirror holder (item 84) by holding the protruding screw and loosening the outer ring (Fig. 14 in the visual assembly guide). Insert a 400–750-nm mirror (item 32) into the groove and screw the outer ring to secure in place (Fig. 15 in the visual assembly guide). Repeat the same for the remaining seven 400–750-nm mirror holders and mirrors.

! CAUTION Handle all optical components carefully to avoid fingerprints or scratches and make sure that no lasers are connected or turned on for Steps 33–163.

33 Screw one of the assemblies formed in Step 32 to one of the assemblies formed in Step 31 (Fig. 16 in the visual assembly guide). Repeat the same for the remaining 14 assemblies formed in Steps 31 and 32.

34 Slot one of the assemblies formed in Step 33 to one of the post holders added to the microscope in Step 30. Repeat the same for the remaining seven assemblies and seven post holders (Fig. 17 in the visual assembly guide).

35 Slot an M6 × 16 mm cap screw into a 50-mm post holder. Screw and tighten the cap screw into the thread on the post holder by using the 5-mm L-shaped hex key with the green band. Repeat the same for another three 50-mm post holders. Screw the four post holders onto the breadboard at positions (18,6), (18,11), (18,16) and (18,21).

36 Screw a 30-mm-long, 12.7-mm-wide optical post (item 73) to a kinematic mirror mount (item 79) (Fig. 18 in the visual assembly guide). Repeat the same for the remaining three optical posts and kinematic mirror mounts.

37 Loosen the set screw on the groove of a kinematic mirror mount assembly formed in Step 36 by using the 2-mm L-shaped hex key with the yellow band (item 99). Insert the 805-nm long-pass dichroic mirror (item 30) in the groove of the kinematic mirror mount and screw the set screw to fix the mirror in place (Fig. 19 in the visual assembly guide). Ensure that the non-reflective surface of the dichroic mirror is facing in the direction of the two black capped knobs on the kinematic mirror mount. The reflective surface can be identified by looking on both sides of the dichroic mirror at an angle (check the following website for more information: https://www.thorlabs.com/newgrouppage9.cfm?objectgroup_id=3313). Repeat the same for the 425-, 505- and 605-nm dichroic mirrors (items 27, 28 and 29, respectively) and the remaining three assemblies formed in Step 36. Note which of the assemblies holds the 425-, 505-, 605- and 805-nm dichroic mirrors.

38 Slot the 425-, 505-, 605- and 825-nm dichroic mirror-holding assemblies formed in Step 37, in order starting from the furthest and working toward the front, into the breadboard-screwed post holders added in Step 35 (Fig. 20 in the visual assembly guide).

39 Loosen the four set screws on the cage plate of the fiber launch system (item 66) by using the 2-mm L-shaped hex key with the yellow band to remove it (Fig. 21 in the visual assembly guide). Loosen and remove the four rods screwed to the XY translation mount of the fiber launch system (Fig. 22 in the visual assembly guide).

40 Loosen the four set screws on the sides of the Z translation mount fixed to the fiber launch system by using the 1.3-mm L-shaped hex key with the orange band (item 99) to remove it (Fig. 23 in the visual assembly guide). This results in two components, an XY translation mount and a Z translation mount.

41 Loosen and remove the SM1 ring inside the XY translation mount by using the SM1 spanner wrench (item 98) (Figs. 24 and 25 in the visual assembly guide).

42 Screw the aspheric lens (item 36) to the extended RMS adapter delivered with the fiber launch system (Fig. 26 in the visual assembly guide).

43 Screw the RMS adapter delivered with the fiber launch system to the assembly formed in Step 42 (Fig. 27 in the visual assembly guide).

44 Screw the assembly formed in point 43 to the XY translation mount. Ensure that the protruding part of the assembly formed in Step 43 is pointing toward the rods fixed on the XY translation mount. Screw the SM1 ring on top of the assembly formed in Step 43 by hand, followed by the SM1 spanner wrench, to secure it in place (Fig. 28 in the visual assembly guide).

45 Loosen and remove the SM1 ring inside the Z translation mount by using the SM1 spanner wrench (Fig. 29 in the visual assembly guide).

46 Screw the FC/PC fiber adapter plate delivered with the fiber launch system to the Z translation mount by using the adjustable spanner wrench delivered with the fiber launch system (Fig. 30 in the visual assembly guide). Ensure that the protruding part of the FC/PC fiber adapter plate is pointing...
Stage 5: first partial assembly of the microscope box ● **Timing 1 h**

▲ **CRITICAL** It is critical that all screws used in Steps 50–200 be strongly tightened (unless otherwise stated) to guarantee high stability of the instrument during imaging.

50 Take the bottom plate of the microscope box on which the number ‘4’ is engraved (item 132) and place it somewhere empty on the breadboard (item 64) with the engraved number facing upward and toward the front. Insert a 6-mm dowel in one of the side holes on the top surface of the plate (Fig. 35 in the visual assembly guide). Repeat with the three other side holes on the top surface of the plate (Fig. 36 in the visual assembly guide).

51 Take the side plate of the microscope box on which the number ‘3’ is engraved (item 132) and seat it upright over the two dowels on the right-hand side of the bottom plate (Fig. 37 in the visual assembly guide). Ensure that the engraved number is at the bottom/front corner facing left.

52 Take the side plate of the microscope box on which the number ‘2’ is engraved (item 132) and seat it over the two dowels on the left-hand side of the bottom plate (Fig. 38 in the visual assembly guide). Ensure that the engraved number is at the bottom/front corner facing right.

53 Flip the assembly formed in Step 52 backward at 90 degrees so that the underside of the bottom plate now faces the front.

54 Screw an M6 × 20 mm cap screw in one of the side counter bores on the now front-facing surface of the bottom plate. Tighten the screw by using the 5-mm L-shaped hex key with the green band (item 99) (Fig. 39 in the visual assembly guide). Repeat the same for all the five remaining side counter bores on the front-facing surface of the bottom plate (Fig. 40 in the visual assembly guide).

55 Flip the assembly formed in Step 54 forward at 90 degrees so that the bottom plate now faces downward again. Ensure that the bottom plate is correctly positioned on the breadboard so that the four central counter bores on the top surface of the plate are perfectly aligned with the breadboard at positions (34,4), (34,8), (41,4) and (41,8).

56 Screw an M6 × 20 mm cap screw in one of the central counter bores at the top surface of the bottom plate (Fig. 41 in the visual assembly guide). Tighten the screw by using the 5-mm L-shaped hex key with the green band. Repeat the same for the remaining three central counter bores at the top surface of the bottom plate.

Stage 6: assembling the excitation module and focus-stabilization system ● **Timing 2 h**

57 Remove the plastic stickers wrapped around four of the rod adapters (item 78). Screw the four rod adapters to the four threads on the right-facing surface on the right-hand side plate.

58 Strongly tighten the plate-fixed rod adapters by inserting the 1.5-mm L-shaped hex key with the purple band (item 99) through the two holes of one adapter and firmly turning (Fig. 42 in the visual assembly guide). Repeat the same for the remaining three rod adapters.

59 Loosen the small screw on one side of a cage cube with dichroic filter mount (item 81) by using the 1.5-mm L-shaped hex key with the purple band (Fig. 43 in the visual assembly guide). Repeat the
same with the other small screw on the adjacent side of the cage cube with dichroic filter mount.

Place the two screws on a flat surface with their heads facing downward.

60 Pull apart the mounting base and cage cube.

**CAUTION** Step 61 requires two individuals. Wear gloves before handling optical components.

61 Press the two clips on the mounting base and gently insert the 700-nm short-pass dichroic beam splitter (item 26) with the engraved ‘Semrock’ logo facing the ‘THORLABS’ logo printed on the mounting base (Fig. 44 in the visual assembly guide). Gently remove your fingers from the clips and from the dichroic beam splitter to secure it in place.

62 Place the mounting base into the cage cube. Ensure that the two white dots on the mounting base are aligned with the two white dots on the cage cube to ensure correct insertion (Fig. 45 in the visual assembly guide). Hold the inserted mounting base and cage cube tight.

63 Screw the two small screws removed in Step 59 to the cage cube. Tighten the screws by using the 1.5-mm L-shaped hex key with the purple band.

64 Take four 0.25-inch rods (item 77). The rods are delivered with a set screw mounted on each end. For one of the four rods, loosen only one set screw by using the 1.3-mm L-shaped hex key with the orange band (item 99) (Fig. 46 in the visual assembly guide). Given the small size of the rod, you might need to wear a latex glove, if available, to grip the rod while loosening the set screw. Repeat the same for the other three rods.

65 Screw one of the rods to one of the corner threads on a specific side of the cage cube with dichroic filter mount. To find the correct side, hold the cage cube with dichroic filter mount with your left hand and ensure that the protruding part is pointing downward and that the dichroic beam splitter inside the assembly is pointing toward the left and front sides (Fig. 47 in the visual assembly guide). Repeat the same with the other three rods, screwing them to the other three corner threads on the same side of the cage cube with dichroic filter mount.

66 Insert the four rods now attached to the cage cube with dichroic filter mount into the four rod adapters on the right-hand side plate (Fig. 48 in the visual assembly guide). Ensure proper insertion by first loosening all the set screws on all four rod adapters by using the 2-mm L-shaped hex key with the yellow band (item 99), then inserting the cage cube with dichroic filter mount and finally tightening all the eight set screws on all the four rod adapters by using the 2-mm L-shaped hex key with the yellow band to firmly secure the assembly in place.

67 Take four 0.5-inch rods (item 76). Repeat Step 64 with these four rods.

68 Screw the four rods to the four corner threads on the right-hand side of the newly mounted cage cube with dichroic filter mount (Fig. 49 in the visual assembly guide).

69 Take a right-angle kinematic mirror mount (item 80) and loosen the set screw on the side of the diagonal face by using the 2-mm L-shaped hex key with the yellow band. Insert a 400–750-nm mirror (item 32) into the groove on the diagonal face of the right-angle kinematic mirror mount. Ensure that the frosted side of the mirror (i.e., the one with the ‘THORLABS’ logo) is facing in the same direction as the two silver knobs on the right-angle kinematic mirror mount. Carefully tighten the set screw by using the 2-mm L-shaped hex key with the yellow band (Fig. 50 in the visual assembly guide).

70 Loosen all eight set screws on the two triangular sides of the right-angle kinematic mirror mount by using the 2-mm L-shaped hex key with the yellow band (Fig. 51 in the visual assembly guide).

71 Slide the right-angle kinematic mirror mount into the four 0.5-inch rods on the right-hand side of the cage cube with dichroic filter mount (Fig. 52 in the visual assembly guide). Ensure that the frosted side of the mirror on the right-angle kinematic mirror mount is facing downward. Tighten all the set screws on the mirror mount assembly by using the 2-mm L-shaped hex key with the yellow band to secure it in place.

72 Screw the rotation mount (item 85) into the SM1 thread in the center of the top side of the right-angle kinematic mirror mount (Fig. 53 in the visual assembly guide).

73 Loosen and remove the SM1 ring threaded inside the rotation mount by using the SM1 spanner wrench (item 98). Insert the 390/482/564/640-nm band-pass filter (item 21) into the groove wherefrom the SM1 ring was removed. Screw and tighten the removed SM1 ring into the SM1 thread inside the rotation mount first by hand and then by using the SM1 spanner wrench (Fig. 54 in the visual assembly guide).

74 Screw the fiber collimator (item 2) into the SM1 thread on the rotation mount (Fig. 55 in the visual assembly guide).
75 Remove the black cap on the other end of the fiber cable (item 1). Insert the bare end to the protruding part on the fiber collimator. Ensure that the fiber cable is properly inserted into the protruding part by aligning the notch on the bare end of the fiber cable with the groove on the protruding part of the fiber collimator (Fig. 56 in the visual assembly guide). Screw and hand-tighten the female metallic barrel on the bare end of the fiber cable with the male screw on the protruding part of the fiber collimator.

76 Take four 0.5-inch rods. Repeat Step 64 with these four rods.

77 Screw the four rods to the four corner threads on the back side of the cage cube with dichroic filter mount.

78 Take a right-angle kinematic mirror mount and loosen the set screw on the side of the diagonal face by using the 2-mm L-shaped hex key with the yellow band. Insert a 750–1,100-nm mirror (item 33) into the groove on the diagonal face of the right-angle kinematic mirror mount. Ensure that the frosted side of the mirror is facing in the same direction as the two silver knobs on the right-angle kinematic mirror mount. Finger-tighten the set screw by using the 2-mm L-shaped hex key with the yellow band.

79 Loosen all eight set screws on the two triangular sides of the right-angle kinematic mirror mount by using the 2-mm L-shaped hex key with the yellow band.

80 Slide the right-angle kinematic mirror mount into the four 0.5-inch rods on the back side of the cage cube with dichroic filter mount (Fig. 57 in the visual assembly guide). Ensure that the frosted side of the mirror on the right-angle kinematic mirror mount is facing backward. Finger-tighten the set screws on the top and bottom sides of the right-angle kinematic mirror mount closest to the front side by using the 2-mm L-shaped hex key with the yellow band to secure it in place.

81 Take four 0.5-inch rods. Repeat Step 64 with these four rods.

82 Hold the non-polarizing beam splitter (item 38) so that the two arrows printed on the splitter are pointing toward the left and front sides. Screw the four rods to the four corner threads on the left-hand side of the non-polarizing beam splitter.

83 Insert the four rods attached to the non-polarizing beam splitter into the four counter bores on the right-hand side of the right-angle kinematic mirror mount (Fig. 58 in the visual assembly guide). Tighten the four set screws on the top and bottom sides of the right-angle kinematic mirror mount closest to the right-hand side by using the 2-mm L-shaped hex key with the yellow band to secure it in place.

84 Loosen and remove one of the two rings screwed to the SM1 coupler (item 88) (Fig. 59 in the visual assembly guide).

85 Screw the SM1 coupler to the SM1 thread on the back side of the non-polarizing beam splitter (Fig. 60 in the visual assembly guide). Tighten the ring on the SM1 coupler to secure the coupler to the non-polarizing beam splitter.

86 Take a rotation mount. Loosen and remove the SM1 ring threaded in the rotation mount by using the SM1 spanner wrench. Insert the neutral density filter (item 37) into the groove wherefrom the SM1 ring was removed (Fig. 61 in the visual assembly guide). Screw and tighten the removed SM1 ring to the SM1 thread inside the rotation mount first by hand and then by using the SM1 spanner wrench.

87 Screw and tighten the rotation mount to the SM1 coupler.

88 Screw the compact diode laser (item 8) to the rotation mount (Fig. 62 in the visual assembly guide). Rotate the laser so that the engraved word ‘CLOSED’ faces upward. Tighten the set screw on the rotation mount by using the 2-mm L-shaped hex key with the yellow band to secure it in place.

89 Remove the circular transparent plastic film on the active region of the lateral effect position sensor (item 10).

90 Screw the SM05-to-SM1 adapter (item 91) to the lateral effect position sensor (Fig. 63 in the visual assembly guide).

91 Loosen and remove the SM1 ring threaded inside a 0.3-inch SM1 lens tube (item 94) by using the SM1 spanner wrench. Insert the 700-nm long-pass filter (item 31) into the groove of the SM1 lens tube with the arrow on the outer edge of the filter pointing away from the groove (Fig. 64 in the visual assembly guide). Screw and tighten the removed SM1 ring to the SM1 lens tube first by hand and then by using the SM1 spanner wrench.

92 Screw the SM1 lens tube to the SM1 thread on the right-hand side of the non-polarizing beam splitter (Fig. 65 in the visual assembly guide). Screw the 25.4-mm focal-length plano convex lens (item 145) to the SM1 lens tube. Loosen and remove the SM1 ring threaded inside a 1-inch SM1
lens tube (item 146) by using the SM1 spanner wrench. Screw the 1-inch SM1 lens tube to the lens tube mounting the plano convex lens.

93 Screw an SM1 coupler to the SM1 lens tube. Tighten the ring on the left-hand side of the SM1 coupler to secure it in place (Fig. 66 in the visual assembly guide).

94 Screw the lateral effect position sensor assembly to the SM1 coupler. Rotate the lateral effect position sensor through two full turns until the protruding wire is pointing upward (Fig. 67 in the visual assembly guide). Rotate and firmly tighten the ring on the right-hand side of the SM1 coupler to secure the lateral effect position sensor in place.

Stage 7: second partial assembly of the microscope box  ● Timing 1 h

95 Take the top plate of the microscope box on which the number ‘1’ is engraved (item 132) and place it on an empty space on the breadboard (item 64) with the engraved number facing upward and toward the front.

96 Take four 0.5-inch rods (item 76). Repeat Step 64 with four of these rods.

97 Screw a rod to one of the threads at the center of the plate (Fig. 68 in the visual assembly guide). Repeat the same for the remaining three threads.

98 Loosen the small screw on one side of a cage cube with dichroic filter mount (item 81) by using the 1.5-mm L-shaped hex key with the purple band (Fig. 43 in the visual assembly guide). Repeat the same with the other small screw on the adjacent side of the cage cube with dichroic filter mount. Place the two screws on a flat surface with their heads facing downward.

99 Pull apart the mounting base and cage cube.

! CAUTION Step 100 requires two individuals.

100 Press the two clips on the mounting base and gently insert the 405/488/561/635/800–1,050-nm super-resolution dichroic beam splitter (item 20) with the engraved dot mark facing the ‘THORLABS’ logo printed on the mounting base (Fig. 69 in the visual assembly guide). Gently remove your fingers from the clips and from the dichroic beam splitter to secure the dichroic beam splitter in place.

101 Place the mounting base into the cage cube. Ensure that the two white dots on the mounting base are aligned with the two white dots on the cage cube to ensure correct insertion. Hold the inserted mounting base and cage cube tight.

102 Screw the two small screws removed in Step 98 to the cage cube. Tighten the screws by using the 1.5-mm L-shaped hex key with the purple band.

103 Hold the cage cube fixed to the dichroic filter mount with your left hand and ensure that the dichroic beam splitter contained within faces upward and to the left-hand side.

104 Remove the plastic stickers wrapped around four of the rod adapters (item 78). Screw the four rod adapters to the four corner threads on the top side of the cage cube with dichroic filter mount. Strongly tighten the rod adapters by inserting the 1.5-mm L-shaped hex key with the purple band through the two holes of one of the four adapters and turning firmly. Repeat for the remaining three rod adapters.

105 Loosen all the set screws on the rod adapters by using the 2-mm L-shaped hex key with the yellow band (item 99) (Fig. 70 in the visual assembly guide). Slide the four rod adapters on the cage cube with dichroic filter mount onto the four rods attached to the top plate (Fig. 71 in the visual assembly guide). Ensure full insertion of the rods into the rod adapters. Tighten the set screws on the rod adapters by using the 2-mm L-shaped hex key with the yellow band.

106 Screw the achromatic lens (item 34) to the SM1 thread on the left side of the cage cube with dichroic filter mount (Fig. 72 in the visual assembly guide).

107 Screw an SM1 end cap (item 87) to the SM1 thread on the right side of the cage cube with dichroic filter mount (Fig. 73 in the visual assembly guide).

108 Take four 0.5-inch rods. Repeat Step 64 with these four rods.

109 Screw the four rods to the four corner threads on the top side of the cage cube with dichroic filter mount (Fig. 74 in the visual assembly guide).

110 Take a right-angle kinematic mirror mount (item 80). Loosen all the set screws on the right-angle kinematic mirror mount by using the 2-mm L-shaped hex key with the yellow band. Slide the right-angle kinematic mirror mount onto the four rods on the top side of the cage cube with dichroic filter mount (Fig. 75 in the visual assembly guide). Ensure that the two silver knobs on the right-angle kinematic mirror mount are pointing upward and toward the front. Tighten the four set
screws on the two triangular faces at the bottom by using the 2-mm L-shaped hex key with the yellow band to secure it in place.

111 Insert a 400–750-nm mirror (item 32) into the groove on the diagonal face of the right-angle kinematic mirror mount. Ensure that the frosted side of the mirror (i.e., the one with the 'THORLABS' logo) is facing in the same direction as the two silver knobs on the right-angle kinematic mirror mount (Fig. 76 in the visual assembly guide). Tighten the set screw on the diagonal face of the right-angle kinematic mirror mount by using the 2-mm L-shaped hex key with the yellow band.

112 Loosen and remove the SM1 ring threaded into a 0.3-inch SM1 lens tube by using the SM1 spanner wrench (item 98). Insert the 446/523/677-nm band-pass filter (item 22) into the groove of the SM1 lens tube. Screw and tighten the removed SM1 ring in the SM1 lens tube first by hand and then by using the SM1 spanner wrench.

113 Screw the SM1 lens tube into the SM1 thread on the back side of the right-angle kinematic mirror mount.

114 Insert four 6-mm dowels (item 116) in the holes on the top edges of the left- and right-side microscope box plates (Fig. 77 in the visual assembly guide).

115 Carefully flip the top microscope box plate upside down and place it on top of the four dowels (Fig. 78 in the visual assembly guide). Gently tap, by using your hands, on the plate until fully secure.

116 Insert six M6 × 20 mm cap screws (item 101) in the counter bores on the top face of the top microscope box plate. Tighten the screws by using the 5-mm L-shaped hex key with the green band (item 99) (Fig. 79 in the visual assembly guide).

117 Take the back plate of the microscope box on which the number '5' is engraved (item 132) and place it on an empty space on the breadboard with the engraved number facing upward and toward the front.

118 Take four 0.25-inch rods. Repeat Step 64 with these four rods.

119 Screw the four rods to the four threads on the top side of the back plate (Fig. 80 in the visual assembly guide).

120 Take the four-position slider (item 67) and orient it so that the black slider is facing upward and the connector on the red electronic board is facing the front. Roughly align the four silver threads on the red electronic board on top of the four rods screwed to the back plate. Slide the black slider to the left so that the two rightmost silver threads on the red electronic board are accessible. Screw two 4-40 × 3/16-inch cap screws (item 100) to the two rightmost silver threads. Finger-tighten the screws by using the Hex 3/32-inch L-shaped hex key (item 100) (Fig. 81 in the visual assembly guide). Slide the black slider to the right so that the two leftmost silver threads on the red electronic board are accessible. Screw two 4-40 × 3/16-inch cap screws to the two leftmost threads. Tighten the screws by using the Hex 3/32-inch L-shaped hex key.

121 Loosen and remove the SM1 rings threaded in three 0.3-inch SM1 lens tubes by using the SM1 spanner wrench. Insert the 525/30-nm, 600/37-nm and 676/29-nm band-pass filters (items 23, 24 and 25, respectively) into the grooves of the three SM1 lens tubes. Screw and tighten the removed SM1 rings inside the three SM1 lens tubes first by hand and then by using the SM1 spanner wrench.

122 Screw the SM1 lens tubes containing the 525/30-nm, 600/37-nm and 676/29-nm band-pass filters to the second, third and fourth SM1 threads (counting from the left-hand side) on the four-position slider (Fig. 82 in the visual assembly guide).

123 Plug the ribbon cable into the off-white connector on the red electronic board (Fig. 83 in the visual assembly guide).

124 Insert four 6-mm dowels in the holes on the back edges of the top and bottom plates of the microscope box.

125 Slide the back plate onto the four dowels with the four-position slider facing the front. Ensure that the ribbon protruding from the four-position slider is inserted into the thin groove formed between the bottom and right plates of the microscope box (Fig. 84 in the visual assembly guide). Ensure that the holes on the back plate are aligned with the dowels. Gently tap, by using your hands, on the back plate until fully secure.

126 Insert 10 M6 × 20 mm cap screws in the counter bores on the back side of the back plate. Tighten the screws by using the 5-mm L-shaped hex key with the green band.
Stage 8: assembling the emission module ● **Timing 1 h**

127 Take four 0.5-inch rods (item 76). Repeat Step 64 with these four rods.
128 Screw the four rods to the four threads on the back side of the back plate (Fig. 85 in the visual assembly guide).
129 Loosen all the set screws on the sides of a cage plate (item 82) by using the 2-mm L-shaped hex key with the yellow band (item 99). Slide the cage plate onto the four rods on the back plate (Fig. 86 in the visual assembly guide). Tighten the screws by using the 2-mm L-shaped hex key with the yellow band.
130 Loosen and remove the two SM1 rings threaded to the cage plate by using the SM1 spanner wrench. Screw an SM1-to-SM2 adapter (item 89) to the cage plate (Fig. 87 in the visual assembly guide).
131 Screw the tube lens (item 35) to the SM1-to-SM2 adapter. Ensure that the arrow printed on the tube lens is pointing toward the front (i.e., toward the microscope box) (Fig. 88 in the visual assembly guide).
132 Screw an SM1-to-SM2 adapter to the tube lens (Fig. 89 in the visual assembly guide).
133 Loosen and remove the two SM1 rings screwed to the SM1 lens tube without external threads (item 95) by using the SM1 spanner wrench.
134 Screw the SM1 lens tube without external threads to the SM1-to-SM2 adapter (Fig. 90 in the visual assembly guide).
135 Screw the SM1 zoom housing (item 86) to the SM1 lens tube without external threads (Fig. 91 in the visual assembly guide).
136 Loosen and remove the SM1 ring threaded to a 2-inch SM1 lens tube (item 93) by using the SM1 spanner wrench.
137 Screw the SM1 lens tube to the SM1 zoom housing (Fig. 92 in the visual assembly guide).
138 Loosen and remove the SM1 ring threaded to a rotation mount (item 85) by using the SM1 spanner wrench.
139 Screw the rotation mount to the SM1 lens tube (Fig. 93 in the visual assembly guide).
140 Screw the SM1-to-C-mount adapter to the rotation mount (Fig. 94 in the visual assembly guide).
141 Remove the yellow plastic film on the active region of the sCMOS camera (item 14).
142 Screw the sCMOS camera to the SM1-to-C-mount adapter (Fig. 95 in the visual assembly guide).
143 Rotate the barrel on the SM1 zoom housing to adjust the distance between the active region of the sCMOS camera and the middle of the tube lens to 15 cm as measured by a ruler (Fig. 96 in the visual assembly guide).
144 Tighten the set screw on the barrel of the SM1 zoom housing by using the 1.3-mm L-shaped hex key with the orange band (item 99) (Fig. 97 in the visual assembly guide).
145 Loosely screw a stainless steel screw (item 102) halfway through the thread on the smaller base of the 2-inch pedestal pillar post (item 71).
146 Rotate the sCMOS camera so that the power switch is on the top left corner. Screw the pedestal pillar post on the right side of the sCMOS camera (Fig. 98 in the visual assembly guide).
147 Rotate the sCMOS camera so that the attached pedestal pillar post sits flat on the breadboard (item 64) (and the power switch is on the bottom left corner).
148 Slide the clamping fork (item 75) on the base of the pedestal pillar post. Insert an M6 × 20 mm cap screw into the groove of the clamping fork. Tighten the cap screw by using the 5-mm L-shaped hex key with the green band (item 99) (Fig. 99 in the visual assembly guide).

Stage 9: assembling the sample stage ● **Timing 2 h**

149 Insert two M4 dowels (item 117) in the holes on the top plate of the microscope box (Fig. 100 in the visual assembly guide).
150 Take the positioner base (item 131) in one hand and hold it so that the counter bores are facing upward. Insert an M1.6 × 20 cap screw (item 118) into one of the six counter bores on the back left corner of the top side of the positioner base (Fig. 101 in the visual assembly guide). Take one of the three positioners (item 15) with the other hand and hold it so that the face of the sliding rail of the positioner is pointing downward. Bring the positioner underneath the positioner base, aligning the inserted screw with the thread on the back left corner of the top surface of the positioner. Tighten the cap screw into the thread by using the T5 screwdriver (item 124) (Fig. 102 in the visual assembly guide). Repeat the same with the remaining five counter bores on the back middle, back
right, front left, front middle and front right corners of the positioner base (Fig. 103 in the visual assembly guide).

151 Flip the positioner base upside down and slide it onto the two M4 dowels inserted in the top plate of the microscope box. Shuffle the positioner base while pushing down, to ensure that it is fully secure. Ensure that the wire protruding from the positioner is pointing toward the left. Insert four M4 × 25 mm cap screws (item 126) into the counter bores on the top side of the positioner base (Fig. 104 in the visual assembly guide). Tighten the cap screws to the top plate of the microscope box by using the 3-mm L-shaped hex key with the light blue band (item 99).

152 Take one of the two V-shaped elements (item 122) and orient it so that the protruding teeth are pointing to the right-hand side and downward. Fix the V-shaped element to the fifth thread (counting from the left-hand side) on the sliding rail of the positioner by using the supplied M2 cap screw (item 122). Take care not to overtighten the screw. Take the second of the three positioners and orient it so that the face of the sliding rail is pointing upward and perpendicular to the first positioner, with its protruding wire pointing to the back. Insert the teeth protruding from the fixed V-shaped element into the third and fifth grooves on the left side of the base of the second positioner (Fig. 105 in the visual assembly guide). Take the other V-shaped element and orient it so that it opposes the existing one. Fix the second V-shaped element to the fifth thread (counting from the right-hand side) on the sliding rail of the first positioner by using the supplied M2 screw. Insert the teeth protruding from the second V-shaped element into the third and fifth grooves on the right side of the base of the second positioner (Fig. 106 in the visual assembly guide). Tighten the two M2 screws by using the 1.5-mm L-shaped hex key with the purple band (item 99).

153 Take the planar connecting element (item 122) and orient it so that its longer base is upright and facing to the front and the shorter base is facing downward, toward the two fixed positioners. Align the two holes on the shorter base of the planar connecting element with the second and third threads (counting from the front edge) on the sliding rail of the second positioner (Fig. 107 in the visual assembly guide). Note that the odd-numbered holes are threaded and that the even-numbered holes are dowel holes. Screw and tighten two of the supplied M2 cap screws through the shorter base of the planar connecting element and into the referred-to threads on the second positioner by using the 1.5-mm L-shaped hex key with the purple band (Fig. 108 in the visual assembly guide).

154 Take the third positioner and orient it so that the face of the sliding rail is pointing downward. Take the rectangular connecting element (item 123) and orient it so that the wider base faces the top side of the positioner. Orient and place the rectangular connecting element so that it is flush with the top side of the positioner (Fig. 109 in the visual assembly guide).

155 Insert one of the supplied M2 cap screws through the back right groove on the rectangular connecting element and into the thread on the back right corner of the third positioner. Tighten the cap screw by using the 1.5-mm L-shaped hex key with the purple band (Fig. 110 in the visual assembly guide). Repeat the same with the remaining five cap screws, positioning them in the back middle, back left, front right, front middle and front left threads on the positioner (Fig. 111 in the visual assembly guide).

156 Rotate and align the top of the rectangular connecting element with the longer base of the planar connecting element so that the third positioner now faces the front. Ensure that the wire protruding from the positioner is pointing upward. Align the first hole (counting from the top) of the longer base of the planar connecting element with the fourth thread (counting from the top) of the rectangular connecting element.

157 Insert one of the supplied M2 cap screws through the first hole (counting from the top) of the longer base of the planar connecting element. Tighten the screw into the fourth thread (counting from the top) of the rectangular connecting element by using the 1.5-mm L-shaped hex key with the purple band (Fig. 112 in the visual assembly guide). Repeat the same with the remaining six M2 cap screws, inserting them through the six holes below the first hole (counting from the top) of the longer base of the planar connecting element (Fig. 113 in the visual assembly guide).

158 Take four 0.5-inch rods (item 76). Repeat Step 64 with these four rods.

159 Screw the four rods to the four threads on the top plate of the microscope box (Fig. 114 in the visual assembly guide).

160 Loosen all the set screws on the sides of a cage plate (item 82) by using the 2-mm L-shaped hex key with the yellow band (item 99). Slide the cage plate onto the four rods. Tighten the set screws on the sides of the cage plate by using the 2-mm L-shaped hex key with the yellow band (Fig. 115 in the visual assembly guide).
Loosen and remove the two SM1 rings threaded to the cage plate by hand or by using the SM1 spanner wrench (item 98).

Screw the SM1-to-M25 adapter (item 90) to the SM1 thread on the top side of the cage plate (Fig. 116 in the visual assembly guide).

Screw the objective lens (item 19) to the M25 thread on the SM1-to-M25 adapter (Fig. 117 in the visual assembly guide).

**Stage 10: setting up electrical connections**

**Timing 2 h**

▲ CRITICAL Before carrying out Steps 164–185, ensure that all equipment is electrically tested as set by institutional or departmental regulations.

▲ CRITICAL The following steps will result in the installation of many cables. Ensure that these cables are managed properly (i.e., prevented from twisting around each other) to keep the equipment tidy. You may want to use the black cable trunk (item 51) for this purpose.

▲ CRITICAL Ensure that all components are turned off before carrying out Steps 164–185.

164 Place the power strip (item 50) at the back left corner of the breadboard (item 64) (Fig. 118 in the visual assembly guide). Fix both ends of the power strip to the breadboard by using two M6 × 20 mm cap screws (item 101). Tighten the screws by using the 5-mm L-shaped hex key with the green band (item 99).

165 Loosen the six screws on the green terminal of the electronic circuit board attached to the ultraviolet (405 nm) laser head (item 5) by using the slotted screwdriver (item 125) (Fig. 119 in the visual assembly guide). Repeat the same for the blue (488 nm) and red (638 nm) laser heads (items 6 and 7, respectively).

166 Insert the bare wires with red and black ribbons (item 57) into the green terminals labeled ‘Vin+’ and ‘Vin−’ on the green terminal of the electronic circuit board attached to the ultraviolet (405 nm) laser head (Fig. 120 in the visual assembly guide). Tighten the two screws adjacent to the inserted wires by using the slotted screwdriver to secure the wires in place. Connect the other end of the wires (i.e., female socket) to the male plug wired to the DC adapter (item 57) (Fig. 121 in the visual assembly guide). Connect the female socket of a power cable (item 60) to the male plug on the DC adapter (Fig. 122 in the visual assembly guide). Connect the female sockets of the three power cables to the first, second and third (counting from the right-hand side) female sockets on the power strip.

167 Cut 2 m from the ribbon cable wheel (item 59) by using a pair of scissors. Separate a group of six wires from the cut ribbon cable (Fig. 123 in the visual assembly guide).

168 Separate 2 cm of each of the six wires from both ends (Fig. 124 in the visual assembly guide). From one end of the cable, separate 1 m into three pairs of wires (Figs. 125 and 126 in the visual assembly guide).

169 Remove 0.5 cm of the insulating material at the end of one wire by using the wire stripper (item 115) (Fig. 127 in the visual assembly guide). Repeat the same for the remaining lanes (Fig. 128 in the visual assembly guide).

170 Insert the bare wires of a pair into the green terminals labeled ‘MOD+’ and ‘MOD−’ of the electronic circuit board attached to the ultraviolet (405 nm) laser head (Fig. 129 in the visual assembly guide). Tighten the two screws adjacent to the inserted wires by using the slotted screwdriver to secure the wires in place. Repeat the same for the blue (488 nm) and red (638 nm) laser heads. Make note of the color of each wire, the corresponding label ‘MOD+/MOD−’ on each green terminal and corresponding laser head. This will be referred to as the connections table.

171 Cut 5 cm from the ribbon cable wheel by using a pair of scissors. Separate three wires individually from the entire length of the cut ribbon cable. Remove 0.5 cm of the insulating material from both ends of each wire by using the wire stripper. Insert the two bare wires of one of the three wires in the two green terminals labeled ‘INT’ of the electronic circuit board attached to the ultraviolet (405 nm) laser head (Fig. 130 in the visual assembly guide). Tighten the two screws adjacent to the inserted wires by using the slotted screwdriver to secure the wires in place. Repeat the same for the blue (488 nm) and red (638 nm) laser heads.

172 Connect the female socket attached to the cable protruding from the green (561 nm) laser head (item 3) to the male plug labeled ‘LD&TEC’ on the laser control box (item 4) (Fig. 131 in the visual assembly guide). Place the control box on the back right corner of the breadboard with the key pointing to the right side. Connect the female socket of a power cable to the male plug on the
control box. Connect the male plug of the power cable to the fourth (counting from the right-hand side) female socket on the power strip.

173 Connect the male plug attached to the cable protruding from the lateral position sensor (item 10) to the female socket labeled ‘DETECTOR IN’ on the auto aligner (item 18) (Fig. 132 in the visual assembly guide). Connect the non-USB male plug of the connection cable (item 18) to the female socket labeled ‘USB’ on the auto aligner (Fig. 133 in the visual assembly guide). Tighten the two screws on the male plug to secure it in place. Connect the male plug on the DC adapter (item 18) with the female socket on the cord extension (item 18) (Fig. 134 in the visual assembly guide). Connect the male plug on the cord extension to the female socket labeled ‘POWER’ on the auto aligner. Ensure that the notch on the male plug is aligned with the groove on the female socket (Fig. 135 in the visual assembly guide). Connect the female socket of a power cable to the male plug on the DC adapter. Connect the male plug of the power cable to the fifth (counting from the right-hand side) female socket on the power strip.

174 Insert the key (item 8) in the lock on the back side of the compact laser diode (item 8) (Fig. 136 in the visual assembly guide). Connect the male plug of the DC adapter (item 18) to the female socket labeled ‘9V, 0.6A’ on the back side of the compact laser diode (Fig. 137 in the visual assembly guide). Insert the appropriate continental plug into the power adapter (Fig. 138 in the visual assembly guide) and connect it to the sixth (counting from the right-hand side) female socket on the power strip. Connect the male micro-USB plug (item 67) into its corresponding female socket on the red control board (Fig. 141 in the visual assembly guide).

175 Connect the female socket of the gray ribbon cable protruding from the right side of the microscope box to the male plug on the red control board (item 67) (Fig. 139 in the visual assembly guide). Ensure that the socket and plug are properly connected by firmly pushing them together. Connect the male plug of the DC adapter (item 67) to the black female socket on the red control board (Fig. 140 in the visual assembly guide). Connect the power plug of the DC adapter to the seventh (counting from the right-hand side) female socket on the power strip. Connect the male micro-USB plug (item 67) to the female socket labeled ‘12V DC SA’ on the back side of the sCMOS camera (Fig. 142 in the visual assembly guide). Connect the power plug of the DC adapter to the eighth (counting from the right-hand side) female socket on the power strip.

176 Connect one of the two male ends of the USB-C cable (item 67) to the female socket labeled ‘USB 3.2 GEN 2/10 Gbps’ on the back side of the sCMOS camera (Fig. 143 in the visual assembly guide). Connect the three male multi-pin plugs protruding from the first, second and third positioners (item 15) to the three female sockets on the MSC2 sensor module (item 16) labeled ‘CH1’, ‘CH2’ and ‘CH3’, respectively (Fig. 144 in the visual assembly guide). Tighten the screws on the male multi-pin plugs to secure them in place. Connect the male multi-pin plug of the MSC2 sensor module to the female socket labeled with the hazard symbol on the MSC2 control system (item 17) (Fig. 145 in the visual assembly guide). Connect the D-type male plug (item 17) into the female socket labeled ‘USB’ on the MSC2 control system (Fig. 146 in the visual assembly guide). Connect the male plug of the DC adapter (item 17) to the female socket on the MSC2 control system (Fig. 147 in the visual assembly guide). Connect the power plug of the DC adapter to the ninth (counting from the right-hand side) female socket on the power strip.

177 Connect one female BNC socket to the BNC male plug of a coaxial cable (item 55) (Fig. 148 in the visual assembly guide). Remove 0.5 cm of the insulating material at the end of each of the four cut wires by using the wire stripper (Fig. 149 in the visual assembly guide). Connect one female BNC socket to the BNC male plug of a coaxial cable (item 55) (Fig. 150 in the visual assembly guide). Repeat the same for the remaining BNC female socket and coaxial cable. Connect the free BNC male plug of one coaxial cable to the BNC female socket on the control box of the green (561 nm) laser head (item 4) (Fig. 151 in the visual assembly guide). Connect the D-type male plug of the other remaining coaxial cable to the BNC female socket on the LED driver (item 54).

178 Connect the green male plug from the LED (item 9) to the female socket labeled ‘LED’ on the LED driver (item 54) (Fig. 152 in the visual assembly guide). Connect the male plug of the DC adapter (item 54) to the female socket labeled ‘DC 15V 1A’ on the LED driver (Fig. 153 in the visual assembly guide). Connect the power plug of the DC adapter to the tenth (counting from the right-hand side) female socket on the power strip.

179 Take the analog digital converter device (item 44) and orient it so that the attached mini-USB female socket is pointing to the left and the board components are facing upward. Loosen all the
181 Refer to the connections table (Step 170). Insert the other end of the wires connected to the terminals labeled ‘MOD−’ and ‘MOD+’ on the electronic circuit board of the ultraviolet (405 nm) laser head to the first and second (counting from the back) green terminals on the analog digital converter device, respectively. Finger-tighten the screws on the green terminals adjacent to the inserted wires by using the slotted screwdriver. Repeat the same for the other ends of the wires connected to the terminals labeled ‘MOD−’ and ‘MOD+’ on the electronic circuit boards of the blue (488 nm) and red (638 nm) laser heads, connecting them to the third, fourth, seventh and eighth (counting from the back) green terminals. Insert the ends of the red and black wires of the male BNC cable connected to the control box of the green (561 nm) laser head to the fifth and sixth (counting from the top) green terminals. Tighten the screws on the green terminals adjacent to the inserted wires by using the slotted screwdriver (Fig. 155 in the visual assembly guide). Connect the mini-USB male plug of one of the two USB connectors (item 48) to the mini-USB female plug on the analog digital converter device (Fig. 156 in the visual assembly guide).

182 Connect the control hub (item 45) as follows:
- Connect one end of each of the two converter device cables (item 49) to the ports labeled ’0’ and ’1’ on the control hub (Fig. 157 in the visual assembly guide).
- Connect the mini-USB male plug of the other USB connector (item 48) to the mini-USB female socket on the control hub (Fig. 158 in the visual assembly guide).
- Using the converter device cables, connect the ports labeled ’1’ and ’0’ on the control hub to the constant DC power source device (item 46) and the variable DC power source device (item 47), respectively (Fig. 159 in the visual assembly guide).
- Loosen all the screws on the green terminals on the constant and variable DC power source devices by using the slotted screwdriver.
- Insert the ends of the red and black wires of the BNC-to-test clip connectors attached to the LED driver to the green terminals labeled ’5V’ and ’GND’ on the constant DC power source device, respectively (Fig. 160 in the visual assembly guide). Tighten the adjacent screws by using the slotted screwdriver to secure the wires in place.
- Cut 1 m from the ribbon cable wheel by using a pair of scissors. Separate two wires from the entire length of the cut ribbon cable. Further separate 2 cm from the individual wires at both ends. Remove 0.5 cm of the insulating material at both ends of each wire by using the wire stripper. Insert one end of the pair of wires into the green terminals, labeled ’+Vout’ and ’GND’, on the variable DC power source device and tighten the screws adjacent to the inserted wires by using the slotted screwdriver to secure them in place. Loosen the screws on the white terminal strip (item 58). Insert the free end of the pair of wires to the two sockets on one side of the terminal strip (Fig. 161 in the visual assembly guide). Tighten the screws adjacent to the inserted wires to secure them in place. Insert the red and blue wires of the vibration motor (item 61) to the other side of the terminal strip (Fig. 162 in the visual assembly guide). Tighten the screws adjacent to the inserted wires to secure them in place. Ensure, under this configuration, that the red wire of the vibration motor is connected to the wire connected to the green terminal labeled ’+Vout’ on the variable DC power source device and that the blue wire of the vibration motor is connected to the wire connected to the green terminal labeled ’GND’.
- Mount the vibration motor on the fiber by using the fiber and vibration motor mount (item 141). The mount comes in two pieces. Each piece contains three grooves: one in the center and two on the sides. Take one piece and insert the vibration motor in the central groove and loop the fiber to insert it in the two side grooves. Secure this arrangement with your fingers. Take the remaining piece of the mount and place it on top of the vibration motor and fiber. Insert two M4 × 20 mm cap screws (item 126) into the side holes of the mount and secure it in place by finger-tightening two M4 washers on each cap screw.

183 Connect the male micro-USB plug of the micro-USB-to-USB-A cable (item 62) to the female socket on the control joystick (item 43) (Fig. 163 in the visual assembly guide).

184 Connecting the USB hub (item 42):
- Connect the male plug of the DC adapter (item 42) to the USB hub.
- Connect the power plug of the DC adapter to the eleventh (counting from the right-hand side) female socket on the power strip.
- Connect the D-type male plug of the USB cable (item 42) to the D-type female socket on the USB hub (Fig. 164 in the visual assembly guide).
Connect the male USB plug of the USB cable to the USB C-to-USB-A adapter (item 41) (Fig. 165 in the visual assembly guide).

Connect the male USB-C plug of the adapter to one of the USB-C sockets on the laptop (item 41).

Connect the other male USB-C plug connected to the sCMOS camera (item 14) to one of the free USB-C sockets on the laptop (item 41).

Connect the male USB-C plug connected to the DC adapter (item 14) to the free remaining USB-C socket on the laptop.

Connect all the six USB-A plugs from the control joystick, control hub, analog digital converter device, MSC2 control system, lateral position sensor and red electronic board of the position slider to the USB hub (Fig. 166 in the visual assembly guide).

Connect the power plug of the DC adapter to the twelfth (counting from the right-hand side) female socket on the power strip.

Stage 11: assembling the laser-combiner enclosure  ●  Timing 30 min

Screw an M6 × 20 mm set screw (item 101) to one end of a rail (item 130) (Fig. 167 in the visual assembly guide). Repeat the same with the remaining three rails. Screw the four rails to the breadboard (item 64) at positions (1,1), (1,29), (22,1) and (22,29) (Fig. 168 in the visual assembly guide). Ensure, without overtightening, that the rails are square with the breadboard (item 64).

Insert the four smallest PVC panels (item 130) into the grooves of the rails to form the sides of the enclosure. Ensure that the panel with the cut-out slot is inserted between the two rails at the back and that all the wires connected to the lasers are fitted through the slot. Place the largest PVC panel on top of the rails so that the holes on the panel are aligned with the threads on the rails. Fix the large top panel to the rails by using four M6 clamping knobs (item 121) (Fig. 169 in the visual assembly guide).

Stage 12: assembling the sample enclosure  ●  Timing 2 h

Take the largest rectangular Perspex panel (item 133) with the semicircular slot and place it along its longer edge on a flat surface. Take one of the two pentagon-shaped Perspex panels (item 133) and place it adjacent to the rectangular panel with the edge containing threads facing the counter bores on one side of the rectangular panel. Insert an M4 × 12 mm cap screw (item 126) into one of the three counter bores and tighten by using the 2.5-mm L-shaped hex key with the burgundy-colored band (item 99) (Fig. 170 in the visual assembly guide). Repeat the same with the two remaining threads along the same edge. Take the remaining pentagon-shaped Perspex panel and place it adjacent to the other edge with counter bores. Insert an M4 × 12 mm cap screw into one of the three counter bores and hand-tighten by using the 2.5-mm L-shaped hex key with the burgundy-colored band. Repeat the same with the two remaining threads along the same edge (Fig. 171 in the visual assembly guide).

Rotate the assembly formed in Step 188 so that the rectangular panel is at the back. Place the narrow rectangular Perspex panel (item 133) containing only four counter bores at the front (Fig. 172 in the visual assembly guide). Insert four M4 × 12 cap screws into the four counter bores and tighten them by using the 2.5-mm L-shaped hex key with the burgundy-colored band.

Place the yet-unused narrow rectangular Perspex panel (item 133) containing several counter bores on top of the assembly (Fig. 173 in the visual assembly guide). Insert five M4 × 12 cap screws into the five counter bores on the top face of the narrow rectangular panel and tighten by using the 2.5-mm L-shaped hex key with the burgundy-colored band. Take one of the two hinges (item 106) and place it on top of the narrow rectangular panel so that two of the counter bores on the hinge are aligned with two of the four threads on the top face of the narrow rectangular panel. Screw a low-profile screw (item 106) into one of the counter bores of the hinge and tighten by using the 2.5-mm L-shaped hex key with the burgundy-colored band (Fig. 174 in the visual assembly guide). Repeat the same with the other counter bore. Repeat the same with the remaining hinge.

Take the large rectangular Perspex panel (item 133) without a hole and place it so that it lies flat on the surface with the counter bores facing down. Take the large rectangular Perspex panel with a hole (item 133) and place it so that one of its longer edges is lying on the flat surface, the hole is in its lowermost position and the three counter bores on the longer side are aligned with the three edge threads on the other panel without a hole. Insert three M4 × 12 mm cap screws into the three counter bores on the longer side of the large rectangular panel with the hole and tighten by using...
the 2.5-mm L-shaped hex key with the burgundy-colored band (Fig. 175 in the visual assembly
guide). Take the triangular Perspex panels (item 133) and place them so that their edge threads are
aligned with the side counter bores on the new assembly. Insert six M4 × 12 mm cap screws into
counter bores on the sides of one of the two rectangular panels and tighten by using the 2.5-mm
L-shaped hex key with the burgundy-colored band. Flip the assembly so that the remaining six free
counter bores are accessible. Insert six M4 × 12 mm cap screws into the counter bores on the sides
of the rectangular panel and tighten by using the 2.5-mm L-shaped hex key with the burgundy-
colored band (Fig. 176 in the visual assembly guide).

192 Flip the assembly formed in Step 191 so that the large rectangular panel with a hole is lying
on the flat surface. Take a handle (item 105) and place it against the threads on the large
rectangular panel. Insert an M5 × 16 mm cap screw (item 126) into an M6 washer (item 101) and
then into one of the counter bores on the handle and tighten by using the 4-mm L-shaped hex key
with the cyan band (item 99) (Fig. 177 in the visual assembly guide). Repeat the same with the other
counter bore.

193 Align the assembly formed in Step 192 with that formed in Step 190 (Fig. 178 in the visual assembly
guide). Align the four free side threads on the assembly formed in Step 192 with the four counter
bores on the hinges of the assembly formed in Step 190. Screw a low-profile screw into one of the
two counter bores of one of the two hinges and tighten by using the 2.5-mm L-shaped hex key with
the burgundy-colored band (Fig. 179 in the visual assembly guide). Repeat the same with the three
remaining counter bores. Gently place the entire assembly (i.e., sample enclosure) on a flat surface.

194 Take a steel angle bracket (item 119) and orient it so that the side with the smaller hole is lying on
the flat surface and that the elongated hole is aligned with one of the four threads on the two
opposite faces of the sample enclosure. Insert a M4 × 8 mm cap screw (item 126) into the elongated
hole of the bracket and screw it into the adjacent thread (Fig. 180 in the visual assembly guide).
Tighten the screw by using the 2.5-mm L-shaped hex key with the brick-red-colored band
(item 99). Repeat the same with three more steel angle brackets.

195 Take the sample enclosure and place it over the microscope box (Fig. 181 in the visual assembly
guide). Align the holes in the four steel angle brackets on the enclosure with the threads on the top
plate of the microscope box. Place an M4 × 8 mm cap screw into one of the four steel angle brackets
and screw it into the adjacent thread on the top plate (Fig. 182 in the visual assembly guide).
Tighten the screw by using the 2.5-mm L-shaped hex key with the brick-red band. Repeat the same
with the other three threads on the top plate.

196 Take four 0.5-inch rods (item 76). Repeat Step 64 with these four rods.

197 Screw the four rods to the four threads on the top panel of the sample enclosure (Fig. 183 in the visual assembly guide).

198 Screw one end of the SM1 lens tube spacer (item 96) to the mounted LED (Fig. 184 in the visual assembly guide). Screw the other end to a cage plate (Fig. 185 in the visual assembly guide). Loosen all the set screws on the sides of the cage plate by using the 2-mm L-shaped hex key with the yellow band (item 99). Slide the cage plate onto the four screwed rods. Tighten the set screws by using the 2-mm L-shaped hex key with the yellow band (Fig. 186 in the visual assembly guide).

Stage 13: assembling and aligning the sample holder ● Timing 30 min

199 Take the sample holder (item 134) and place it so that its longer edge sits flat against the third
positioner. Insert a supplied M2 cap screw (item 14) in one of the two counter bores on the sample
holder. Ensure that the screw is aligned with the sixth thread (eleventh hole, counting from the top)
on the positioner. Note that only the odd-numbered holes are threaded. Loosely screw the cap
screw by using the 1.5-mm L-shaped hex key with the purple band (item 99) (Fig. 187 in the visual assembly guide). Repeat the same with the other counter bore.

200 Place the bullseye level (item 104) on the top plate of the microscope box. Note the position of the
air bubble on the bullseye level as accurately as possible. Place a coverslip (item 40) inside the
groove of the sample holder (Fig. 188 in the visual assembly guide). Secure the coverslip by using
the installed clips. Place the bullseye level on the secured coverslip. Adjust the rotation of the
sample holder until the position of the air bubble on the bullseye level on the coverslip is identical
to the position noted previously. When the positions are matched, tighten the two M2 screws
inserted in the counter bores of the sample holder by using the 1.5-mm L-shaped hex key with the
purple band, checking afterward that the position on the bullseye level has not changed (Fig. 189 in
the visual assembly guide).
Stage 14: switching on the microscope ● **Timing 5 min**

**CAUTION** Wear protective goggles (item 137) from Step 201 to Step 239.

201 To switch on the device for the first time:
- Switch on the main power outlet to which the power strip (item 50) is connected.
- Switch on the power button on the power strip.
- Switch on the power button on the MSC2 control system (item 17).
- Switch on the power button on the sCMOS camera (item 14).
- Switch on the power button on the LED driver (item 54).
- Switch on the power button on the position aligner (item 18).
- Switch on the power button on the green laser control box (item 4).
- Turn on the key on the green laser control box to the position labeled ‘ON’.
- Turn on the key at the back side of the compact diode laser (item 8) (Fig. 1 in the visual operation guide; see Code availability).
- Press the brown button labeled ‘ENABLE’ at the back side of the compact diode laser (Fig. 2 in the visual operation guide).
- Flip the handle labeled ‘LASER APERTURE SHUTTER’ on the compact diode laser to the position labeled ‘OPEN’ (Fig. 3 in the visual operation guide).
- Open and switch on the computer.

? **TROUBLESHOOTING**

202 To switch on the device for subsequent times:
- Switch on the main power outlet to which the power strip (item 50) is connected.
- Press the brown button labeled ‘ENABLE’ at the back side of the compact diode laser (Fig. 2 in the visual operation guide).
- Open and switch on the computer.

? **TROUBLESHOOTING**

Stage 15: setting up the computer and installing the NanoPro 1.0 control software ● **Timing 2 h**

203 Download and install the National Instruments LabVIEW 2020 SP1 64-bit runtime engine from the following link: https://www.ni.com/en-gb/support/downloads/software-products/download.labview.html#369642.

204 Download and install the National Instruments VISA 20.0 from the following link: https://www.ni.com/en-gb/support/downloads/drivers/download.ni-visa.html#346210.

205 Unzip the NanoPro 1.0 software (see Code availability) folder and copy its contents to a new folder on the desktop renamed to ‘NanoPro 1.0’. Ensure that the contents of the unzipped folder are as follows: two folders named ‘data’ and ‘Dependencies’, five files named ‘Configuration.csv’, ‘Sequence.csv’, ‘NanoPro_v1.3.aliases’, ‘NanoPro_v1.3.exe’ and ‘NanoPro_v1.3.ini’.

206 Download and install the Kinesis 64-bit software for 64-bit windows from the following link: https://www.thorlabs.com/Software/Motion%20Control/KINESIS/Application/v1.14.35/kinesis_20468_setup_x64.exe. Copy the contents from the folder path ‘C:\Program Files\Thorlabs\Kinesis’ and paste them into the desktop folder path ‘…\NanoPro 1.0\Dependencies\Thorlabs’.

207 Install the ‘PVCamSDK_Setup.exe’ and ‘PMQI-LabViewSamples_Setup_1.2.2.1.exe’ software files provided with the sCMOS camera (item 14). Copy the files ‘pvcam_helper_-coloured_v1.dll’ and ‘PVCamNET.dll’ from the folder path ‘C:\Program Files\Photometrics\PMQI-LabViewSamples\Examples\2018\Dependencies’ and paste them into the desktop folder path ‘…\NanoPro 1.0\Dependencies\Photometrics’.

208 Request the drivers’ file from SmarAct (supplier of item 15). Unzip the contents of the drivers’ file and install the file ‘CDM21226_Setup.exe’. Install the ‘MCS2_Installer_2.1.3.exe’ software file provided with the MSC2 control system (item 17). During installation, ensure that the check boxes for ‘MSC2 Tools and Programs’ and ‘Support for MSC2 with USB Interface’ are checked.

209 Download and install the 64-bit version of the Phidget control software from the following link: https://www.phidgets.com/downloads/phidget22/libraries/windows/Phidget22-x64.exe. Copy the contents from the folder path ‘C:\Program Files\Phidgets\Phidget22’ and paste them into the desktop folder path ‘…\NanoPro 1.0\Dependencies\Phidgets’.
Stage 16: aligning the laser combiner

210 Download and install ImageJ (item 139) from the following link: https://wsr.imagej.net/distros/win/ij153-win-java8.zip. Download and install ThunderSTORM from the following link: https://zitmen.github.io/thunderstorm/. Follow the installation instructions provided.

211 Open the ‘Configuration.csv’ file in the folder unzipped in Step 205. Edit the file by using the following information:

- Enter the serial number of the analog digital converter device (item 44) in the field opposite from ‘Phidget 1 Serial Number’. The six-digit serial number is printed on a white sticker fixed to the analog digital converter device.
- Enter the serial number of the control hub (item 45) in the field opposite from ‘Phidget 2 Serial Number’. The six-digit serial number is printed on a white sticker fixed to the control hub.
- Enter the serial number of the position aligner (item 18) in the field opposite from ‘Photo Sensitive Detector Serial Number’. The eight-digit serial number is printed on a black sticker fixed to the position aligner.
- Enter the COM port number of the four-position slider (item 67) in the field opposite from the ‘Filter Slider COM port’. To find the COM port number of the slider, type ‘device manager’ in the search box of Windows 10 located at the bottom left corner of the screen. Left-click the ‘Device Manager’ tab that appears under the Best Match list. A long list of components will be shown. Browse to the component named ‘Ports (COM & LPT)’ and left-click the gray arrow on the left side of the component. The COM port number will be dropped down.

212 Run the NanoPro 1.0 control software by double-clicking the ‘NanoPro_v1.3.exe’ software file in the ‘NanoPro 1.0’ folder. The NanoPro 1.0 control software window should appear (Fig. 4 in the visual operation guide) as well as another blank camera view window (Fig. 5 in the visual operation guide). The ‘Ready’ button on the NanoPro 1.0 control software window should display ‘Ready’.

? TROUBLESHOOTING

Stage 16: aligning the laser combiner

213 Familiarize yourself with the control joystick (item 43) (Figs. 6 and 7 in the visual operation guide).

214 Switch on the red laser (item 7) by using the control joystick (Fig. 6 in the visual operation guide). Increase the power of the red laser to 0.5 as indicated on the power sensor in front of the red laser and record the reading on the power meter, hereafter referred to as the first recorded reading (Video 1 at https://github.com/jdanial/NanoPro).

215 Connect the power sensor (item 13) to the power meter (item 12). Switch on the power meter and, if needed, connect to an appropriate power plug. Change the settings on the power meter so that the wavelength is set to 638 nm. Check the manual for instructions on operating the power meter.

! CAUTION Red laser will be emitting at 70 mW. Wear safety glasses (item 137) and do not look directly into the beam. Ensure that the room is secured against uncontrolled entry. Any reflective items (e.g., jewelry and wristwatches) must be removed for alignment.

! CAUTION Do not increase the power of the red laser more than 3.5, to prevent overheating of the laser head under ambient conditions.

216 Loosen the four clamping knobs (item 121) securing the top panel of the laser enclosure assembled in Step 187. Lift the panel and stow it in an appropriate place.

217 Adjust the power meter settings according to the vendor’s instructions. Place the active area of the power sensor in front of the red laser and record the reading on the power meter, hereafter referred to as the first recorded reading (Video 1 at https://github.com/jdanial/NanoPro).

218 Loosen the black capped screw on the post holder (item 74) on the breadboard (item 64) at position (16,4) to liberate the post assembly (hereafter referred to as the first post assembly). Rotate the first post assembly so that the laser beam reflected off the mounted mirror is steered toward the center of the mirror on the post assembly on the breadboard at position (14,7) (hereafter referred to as the second post assembly) (Fig. 1 in the visual alignment guide; see Code availability). Tighten the black capped screw by using the 5-mm L-shaped hex key with the green band (item 99) to secure the first post assembly in place (Video 2 at https://github.com/jdanial/NanoPro). Loosen the black-capped screw on the post holder on the breadboard at position (14,7) to liberate the second post assembly. Rotate the second post assembly so that the laser beam reflected off the mounted mirror is steered 2 mm off the front edge of the 805-nm long-pass dichroic mirror (item 30), which is part of the post assembly on the breadboard at position (18,6) (hereafter referred to as the third post assembly) (Fig. 2 in the visual alignment guide). Tighten the black capped screw by using the 5-mm L-shaped...
hex key with the green band to secure the second post assembly in place (Video 3 at https://github.com/jdanial/NanoPro). Take an A4 piece of paper, fold it in half and rest it in front of the fiber launch system (item 66). Loosen the black capped screw on the post holder on the breadboard at position (18,6) to liberate the third post assembly. Rotate the third post assembly so that the laser beam reflected off the mounted 805-nm long-pass dichroic mirror is steered through all the mounted long-pass dichroic mirrors (items 27, 28 and 29) and toward the folded piece of paper (Fig. 3 in the visual alignment guide). Remove the piece of paper. Tighten the black capped screw by using the 5-mm L-shaped hex key with the green band to secure the third post assembly in place (Fig. 4 in the visual alignment guide and Video 4 at https://github.com/jdanial/NanoPro).

- **Step 219**: Rotate the two knobs on the kinematic mirror mount (item 79) on the third assembly, one after the other, so that the beam is steered toward the center of the fiber launch system (Video 5 at https://github.com/jdanial/NanoPro).
- **Step 220**: Screw the FC/PC fiber adapter (item 97) to the thread on the power sensor (item 13) (Fig. 5 in the visual alignment guide). Attach the fiber end (item 1) screwed to the collimator (item 2) to the FC/PC connector on the fiber adapter (Fig. 6 in the visual alignment guide and Video 6 at https://github.com/jdanial/NanoPro).
- **Step 221**: Rotate the two knobs on the kinematic mirror mount on the third assembly, one after the other, to gradually increase the power reading on the power meter (Fig. 7 in the visual alignment guide). Repeat until the reading on the power meter is maximized. Record the reading on the power meter and proceed to the next step (Video 7 at https://github.com/jdanial/NanoPro).
- **Step 222**: Rotate the lowermost silver knob on the first assembly in one direction a small turn and observe the decrease in the reading on the power meter (Fig. 8 in the visual alignment guide). Remember the direction of rotation. Rotate the lowermost silver knob on the second assembly in one direction a small turn and observe the increase in the reading on the power meter. If the reading on the power meter does not increase, then rotate the knob in the other direction a small turn and observe the increase in the reading on the power meter. Repeat until the reading on the power meter is maximized. Record the reading on the power meter and proceed to the next step.
- **Step 223**: Rotate the uppermost silver knob on the first assembly in one direction a small turn and observe the decrease in the reading on the power meter. Remember the direction of rotation. Rotate the uppermost silver knob on the second assembly in one direction a small turn and observe the increase in the reading on the power meter. If the reading on the power meter does not increase, then rotate the knob in the other direction a small turn and observe the increase in the reading on the power meter. Repeat until the reading on the power meter is maximized. Record the reading on the power meter and proceed to the next step (Video 8 at https://github.com/jdanial/NanoPro).
- **Step 224**: Rotate the black knob pointing up on the fiber launch system to gradually increase the power reading on the power meter (Fig. 9 in the visual alignment guide). Repeat until the reading on the power meter is maximized. Rotate the black knob pointing left on the fiber launch system to gradually increase the power reading on the power meter (Fig. 10 in the visual alignment guide). Repeat until the reading on the power meter is maximized. Record the reading on the power meter and proceed to the next step (Video 9 at https://github.com/jdanial/NanoPro).
- **Step 225**: Loosen all four set screws on the side of the Z translation mount fixed to the fiber launch system by using the 1.3-mm L-shaped hex key with the orange band (Fig. 11 in the visual alignment guide and Video 10 at https://github.com/jdanial/NanoPro). Move the Z translation mount in one direction to gradually increase the power reading on the power meter. If the reading on the power meter does not increase, then move the Z translation mount in the other direction and observe the increase in the reading on the power meter. Repeat until the reading on the power meter is maximized (Video 11 at https://github.com/jdanial/NanoPro). Tighten all four set screws on the side of the Z translation mount fixed to the fiber launch system by using the 1.3-mm L-shaped hex key with the orange band while holding the Z translation mount in place (Video 12 at https://github.com/jdanial/NanoPro). Rotate the black knob pointing back on the fiber launch system to gradually increase the power reading on the power meter. Repeat until the reading on the power meter is maximized (Video 13 at https://github.com/jdanial/NanoPro). Record the reading on the power meter and proceed to the next step.
- **Step 226**: Repeat Steps 221–225 and observe the increase in the reading on the power meter. Repeat until the reading on the power meter is maximized. Record the reading on the power meter, hereafter referred to as the last recorded reading.
- **Step 227**: If the last recorded reading is >75% of the first recorded reading, then proceed to Step 229; otherwise, proceed to the next step.
228 Take an A4 piece of paper, fold it in half and rest it in front of the fiber launch system. Insert the 0.7-mm L-shaped hex key with the red band (item 99) through the two grooves of the external thread on the head of the red laser (Fig. 12 in the visual alignment guide). Rotate the L-shaped hex key in one direction and observe the reduction in the beam size on the folded piece of paper. If the beam is increased in size, rotate the L-shaped hex key in the other direction and observe the reduction in the beam size. Repeat until the beam size is reduced to a minimum. Repeat Steps 221–227.

229 Repeat Steps 214–228 (excluding Steps 216, 223, 224 and 225) to align the green, blue and ultraviolet lasers (in this order), one at a time, noting the following (Videos 14–18 at https://github.com/jdanial/NanoPro):

- In Step 214, switch on the green, blue or ultraviolet laser.
- In Step 215, set the wavelength to 561 nm (for the green laser), 488 nm (for the blue laser) and 405 nm (for the ultraviolet laser).
- In Step 217, place the active area of the power sensor in front of the switched-on laser.
- For the green laser: in Steps 218–233, the first assembly is inserted into the post holder on the breadboard at position (16,9), the second assembly is inserted into the post holder on the breadboard at position (14,12) and the third assembly is inserted into the post holder on the breadboard at position (18,11).
- For the blue laser: in Steps 218–233, the first assembly is inserted into the post holder on the breadboard at position (16,14), the second assembly is inserted into the post holder on the breadboard at position (14,17) and the third assembly is inserted into the post holder screwed to the breadboard at position (18,16).
- For the ultraviolet laser: in Steps 218–233, the first assembly is inserted into the post holder screwed on the breadboard at position (16,19), the second assembly is inserted into the post holder on the breadboard at position (14,22) and the third assembly is inserted into the post holder on the breadboard at position (18,21).
- Step 228 is omitted for the green laser.

230 Place the stowed top panel of the laser enclosure on top of the rails so that the holes on the panel are aligned with the threads on the rails. Fix the large top panel to the rails by using all four clamping knobs.

**CRITICAL STEP** Press the ‘Live’ button on the control joystick to stop the emission from the ultraviolet laser. Ensure that the ‘Ready’ message is displayed in the ‘[Message box]’ on the NanoPro 1.0 control software window.

231 Loosen the fiber end from the FC/PC fiber adapter and fix it back to the collimator.

232 Switch on the red laser by using the control joystick. Increase the power of the red laser to 2.0 as indicated on the ‘[Power level box]’ on the NanoPro 1.0 control software window. Press the ‘Live’ button on the control joystick to activate laser emission. Switch off the lights of the room.

**TROUBLESHOOTING**

233 Rotate the silver knobs on the right-angled kinematic mirror mount (item 80, added in Step 71) so that the red beam comes straight out of the objective (item 19) and onto the ceiling (Fig. 13 in the visual alignment guide). Rotate the red barrel on the collimator (Fig. 14 in the visual alignment guide) so that the beam is reduced in size and eventually squared at the ceiling (Fig. 15 in the visual alignment guide). Rotate the rotation mount (item 85, added in Step 72) (Fig. 16 in the visual alignment guide) so that the beam is square with the microscope box. Tighten the set screw on the collimator by using the 1.3-mm L-shaped hex key with the orange band (Fig. 17 in the visual alignment guide). Tighten the set screw on the rotation mount by using the 2-mm L-shaped hex key (item 99) with the yellow band to secure it in place (Fig. 18 in the visual alignment guide).

**Stage 17: aligning the excitation and emission paths ● Timing 20 min**

234 Squirt two drops of the immersion oil (item 140) on top of the objective (item 19).

235 Mount the 40-nm nanoruler sample (item 135) on the sample holder (item 134) and secure it from both sides by using the clips, with the smaller coverslip facing downward. Ensure that the sample is properly mounted according to the delivered instructions.

236 Switch on the green laser (item 3) by using the control joystick (item 43) (Fig. 6 in the visual operation guide). Increase the power of the green laser to 4.0 as indicated on the ‘[Power level box]’ on the NanoPro 1.0 control software window (Fig. 7 in the visual operation guide). Press the ‘Live’ button on the control joystick to activate laser emission (Fig. 7 in the visual operation guide).

**TROUBLESHOOTING**
237 Rotate the lowermost silver knob on the kinematic mirror mount (item 80, added in Step 71) so that the squared beam emerging out of the objective is inclined toward the left-hand side and until the beam undergoes TIR. TIR is roughly reached when the beam is seen to form three dots at the glass interface of the objective: two on the side and one in the middle. Ensure that the beam is inclined straight to the left and not at an angle by rotating the uppermost silver knob on the kinematic mirror mount.

238 Move the positioners (item 15) downward (coarse) by using the control joystick (Fig. 6 in the visual operation guide) until the immersion oil on top of the objective touches the bottom of the coverslip. Move the positioners downward (coarse) stepwise by using the control joystick until you observe the pattern on the camera view window shown in Fig. 20 in the visual alignment guide. Move the positioners downward or upward (fine) stepwise by using the control joystick until you observe the pattern on the camera view window shown in Fig. 20 in the visual alignment guide. The pattern might not be centered on the camera (Fig. 21 in the visual alignment guide). If this is the case, rotate the silver knobs on the right-angle kinematic mirror mount inside the microscope box (item 80, added in Step 110) to center the pattern on the camera (Fig. 22 in the visual alignment guide). Further adjust the angle of the beam to ensure proper TIR excitation as described in Step 237. The angle where the image shows highest contrast is the angle at which TIR occurs (Fig. 4a–d).

239 Press the ‘Live’ button on the control joystick to de-activate laser emission.

Stage 18: aligning the focus stabilization system  ● Timing 30 min

240 Insert the detector card (item 11) into the microscope box and place the orange active area in front of the achromatic doublet lens (item 34). Observe the bright spot on the detector card resulting from the detection of the infrared beam produced by the compact diode laser (item 8). Rotate the silver knobs on the kinematic mirror mount (item 80, added in Step 80) to roughly center the beam on the achromatic doublet lens.

241 Place the detector card 2 cm away from the objective lens with the orange active area facing downward. Observe the bright spot on the detector card (Fig. 23 in the visual alignment guide).

242 Rotate the uppermost silver knob on the kinematic mirror mount while tracking the bright spot on the detector card as it inclines to the back (Fig. 24 in the visual alignment guide) and until the spot disappears. If it inclines at an angle, rotate the lowermost silver knob to center the bright spot. Ensure that ‘Focused’ is displayed in the ‘[Focus status box]’ (Fig. 4 in the visual operation guide). Observe the auto aligner (item 18). Rotate the uppermost silver knob on the kinematic mirror mount until the white circle on the square screen on the auto aligner is displaced to the center of the y-axis. Move the positioners (item 15) upward and then downward (fine) by using the control joystick (Fig. 6 in the visual operation guide). Ensure that the white circle moves in a straight line along the y-axis as the positioners are moved.

243 Take the front plate of the microscope box on which the number ‘6’ is engraved (item 132) and use it to cover the front opening of the microscope box (Fig. 190 in the visual assembly guide).

244 Insert an M6 × 20 mm cap screw (item 101) into one of the eight counter bores on the front side of the front plate. Screw and tighten the cap screw by using the 5-mm L-shaped hex key with the green band (item 99) (Fig. 191 in the visual assembly guide). Repeat the same with the remaining seven counter bores. This marks the endpoint of the assembly and alignment of the microscope (Fig. 192 in the visual assembly guide).

245 Move the positioners upward 1 cm above the objective lens by using the control joystick. Unmount the sample.

Stage 19: measuring the camera pixel size  ● Timing 30 min

246 Mount the variable line grating (item 39) on the sample holder (item 134) and secure it from both ends by using the clips. Ensure that the variable line grating is mounted with the black inscribing facing downward.

247 Switch on the LED (item 9) by using the control joystick (item 43) (Fig. 6 in the visual operation guide). Increase the power of the LED by rotating the knob on the LED driver (item 54) to halfway between the labels ‘0’ and ‘LIMIT’. Ensure that the switch on the LED driver is flipped to the position labeled ’TRIG’.

? TROUBLESHOOTING
248 Press the 'Live' button on the control joystick to activate laser emission (Fig. 7 in the visual operation guide). Ensure that 'Live' is displayed in the '[Message box]' on the NanoPro 1.0 control software window (Fig. 4 in the visual operation guide).

? TROUBLESHOOTING
249 Move the positioners (item 15) downward (coarse) by using the control joystick (Fig. 6 in the visual operation guide) until the immersion oil on top of the objective touches the bottom of the coverslip. Move the positioners to the left/right and back/front to position the objective lens underneath the furthest inscribed band (counting from the side with the band with four thick lines). Move the positioners (item 15) downward (coarse or fine) by using the control joystick (Fig. 6 in the visual operation guide) to focus the sample as described in Step 238. Observe the pattern on the camera view window shown in Fig. 25 in the visual alignment guide. Press the 'Live' button on the control joystick to de-activate LED emission. Ensure that 'Ready' is displayed in the '[Message box]' on the NanoPro 1.0 control software window.

? TROUBLESHOOTING
250 Set the '[Exposure time]' on the NanoPro 1.0 control software window (Fig. 4 in the visual operation guide) to 50. Set the '[Number of frames]' on the NanoPro 1.0 control software window (Fig. 4 in the visual operation guide) to 1.

251 Create a new folder on the desktop and rename it as 'Pixel'.

252 Select the '[Acquisition path]' on the NanoPro 1.0 control software window (Fig. 4 in the visual operation guide).

253 Press the 'Acquire' button on the control joystick to start acquisition. Ensure that 'Acquire' is displayed in the '[Message box]' on the NanoPro 1.0 control software window. Gently rest the control joystick on the breadboard (item 64) to reduce perturbations to the microscope.

? TROUBLESHOOTING
254 Wait until acquisition is complete. Acquisition is complete when LED emission is automatically de-activated, and 'Ready' is displayed in the '[Message box]' on the NanoPro 1.0 control software window.

? TROUBLESHOOTING
255 Move the positioners upward 1 cm above the objective lens by using the control joystick. Unmount the sample.

256 The acquisition of the variable line grating will be found in the folder named '1' in the folder created in Step 252. Open the '.tif' file contained within by using the installed ImageJ software (item 139). Press the *straight* line tool on the ImageJ toolbar and draw a horizontal line from the middle of the darkest mark on the right of the image to the middle of the eleventh darkest mark (counting from the right of the image) by left-clicking the mouse button on the image and dragging across. Before releasing the mouse button, record the length of the drawn line, in pixels, as displayed in the message bar of ImageJ.

257 To calculate the pixel size (ps), use the following formula: \[ ps_{\text{nm/pixel}} = \frac{40000}{\text{#pixels}}. \]

Stage 20: imaging ground-truth samples to establish performance ● Timing 1 h

258 Take the 40-nm nanoruler sample (item 135) from the refrigerator and leave it in the microscope room for \( \geq 30 \) min for its temperature to equilibrate to prevent excessive drift during acquisition. Mount the 40-nm nanoruler sample on the sample holder (item 134) and secure it from both ends by using the clips.

259 Switch on the (561 nm) green laser (item 3) by using the control joystick (item 43) (Fig. 6 in the visual operation guide).

? TROUBLESHOOTING
260 Press the 'Live' button on the control joystick to activate laser emission (Fig. 7 in the visual operation guide). Ensure that 'Live' is displayed in the '[Message box]' on the NanoPro 1.0 control software window (Fig. 4 in the visual operation guide).

? TROUBLESHOOTING
261 Move the positioners (item 15) downward (coarse or fine) by using the control joystick (Fig. 6 in the visual operation guide) to focus the sample as described in Step 238. A small portion of the image detected by the camera will be shown. To see the full image, zoom out by right-clicking on the camera view window (Fig. 5 in the visual operation guide) and left-clicking 'Zoom -' from the drop-down menu several times.

? TROUBLESHOOTING
Activate the autofocus system by using the control joystick (Fig. 6 in the visual operation guide) to lock the focus. Ensure that 'Locked' is displayed in the '[Focus status box]' on the NanoPro 1.0 control software window (Fig. 4 in the visual operation guide).

**TROUBLESHOOTING**

Press the 'Live' button on the control joystick to de-activate laser emission. Ensure that 'Ready' is displayed in the '[Message box]' on the NanoPro 1.0 control software window.

Set the '[Exposure time]' on the NanoPro 1.0 control software window (Fig. 4 in the visual operation guide) to 150. Set the '[Number of frames]' on the NanoPro 1.0 control software window (Fig. 4 in the visual operation guide) to 20,000.

Create a new folder on the desktop and rename it as 'Test'.

Select the '[Acquisition path]' on the NanoPro 1.0 control software window (Fig. 4 in the visual operation guide) and choose the created folder.

Crop the area of the field of view (FOV) by right-clicking on the camera view window and then left-clicking 'Add region' from the drop-down menu. A small green box will appear. Expand the green box by dragging its corners and move it by dragging it from the center so that it covers only the region of interest.

Press the 'Acquire' button on the control joystick to start acquisition. Ensure that 'Acquire' is displayed in the '[Message box]' on the NanoPro 1.0 control software window. Gently rest the control joystick on the breadboard (item 64) to reduce perturbations to the microscope.

**TROUBLESHOOTING**

Wait until acquisition is complete. Acquisition is complete when laser emission is automatically de-activated, and 'Ready' is displayed in the '[Message box]' on the NanoPro 1.0 control software window. Remove the crop on the FOV by right-clicking on the camera view window and then left-clicking 'Delete region' from the drop-down menu.

**TROUBLESHOOTING**

Move the positioners upward 1 cm above the objective lens by using the control joystick. Unmount the 40-nm nanoruler sample and store it in the refrigerator.

Squirt one drop of the immersion oil (item 140) on top of the objective (item 19).

Take the 20-nm nanoruler sample (item 136) from the refrigerator and leave it in the microscope room for ≥30 min for its temperature to equilibrate to prevent excessive drift during acquisition. Mount and secure the 20-nm nanoruler sample as described in Step 235.

Repeat Steps 260–271 (excluding Steps 266–268). The two acquisitions of the 40-nm nanoruler sample and the 20-nm nanoruler sample will be found in the folders named '1' and '2', respectively, in the folder created in Step 266.

**TROUBLESHOOTING**

**Stage 21: data processing** • **Timing 1 h**

Open the installed ImageJ software (item 139).

Drag the folder labeled ‘1’ inside the folder created in Step 266 and drop it on the message bar of ImageJ. A new dialog window will appear. Check the box labeled ‘Use Virtual Stack’ and click the button labeled 'Yes'. Wait for the video to load as indicated in the message bar of ImageJ.

Press 'Plugins' on the menu bar, hover over 'ThunderSTORM' and then hover over 'Run analysis'. A new dialog window will appear. In the container labeled 'Camera', press the button labeled 'Camera setup'. Enter the pixel size calculated in Step 258 in the field labeled 'Pixel size [nm]', enter '0.25' in the field labeled 'Photoelectrons per A/D count' (consult the vendor for the exact figure quoting 100 MHz/12 bit as Readout Speed/Data Bits), enter '100' in the field labeled 'Base level [A/D counts]' and uncheck the box labeled 'EM gain'. In the container labeled 'Approximate localization of molecules', enter 'std(Wave.F1)' in the field labeled 'Approximate localization of molecules'. In the container labeled 'Visualisation of the results', enter '26' in the field labeled 'Magnification' and enter '1000' in the field labeled 'Update frequency [frames]'. Press the button labeled 'Ok'. Wait for the video to be processed as indicated in the message bar of ImageJ. During processing, a window will appear containing the super-resolved image. The super-resolved image is not drift-corrected and, therefore, the nanorulers will not be seen.

Once processing is complete, a new table will appear. The table contains information on each detected burst as well as tools to manipulate each burst. Press the tab labeled 'Drift correction' and press the radio button labeled 'Cross correlation'. Press the double arrow button in front of the radio button, enter '10' in the field labeled 'Number of bins' and enter '26' in the field labeled...
Magnification. Wait for the super-resolved image to be drift-corrected as indicated in the message bar of ImageJ. Once completed, a super-resolved image (Fig. 3a) of the 40-nm nanoruler sample should be seen.

278 Repeat Step 276 with the folder labeled ‘2’ inside the folder created in Step 266.

279 Once processing is complete, a new table will appear. The table contains information on each detected burst as well as tools to manipulate each burst. Press the tab labeled ‘Drift correction’ and

Fig. 4 | Images from good- and poor-quality alignment. a–d, Full field-of-view diffraction-limited images of the ground-truth 40-nm nanoruler sample acquired with good alignment conditions (a), excitation path misaligned (b), emission path misaligned (c) and sample holder tilted (d). e, f, Cropped-view super-resolved images of the 40-nm nanoruler sample acquired with good fiber agitation showing homogeneous illumination (e) and no, or poor, fiber agitation showing dark and bright patches with varying localization densities (f). Scale bars: a–f, 10 µm; insets in e and f, 1 µm.
Stage 22: multi-sample acquisitions ◗ Timing dependent on the sequence of events

▲ CRITICAL Ensure that a spreadsheet software, such as Microsoft Excel, is installed on the computer before proceeding with Steps 281–292.

▲ CRITICAL If a large number of samples is to be imaged, add an extra drop of oil on top of the objective.

280 Enter the number of times (i.e., repeats) the mounted sample is to be imaged in the objective.

281 Enter the number of frames to be imaged for each entered laser in the objective.

282 Enter the distance between each well in the direction parallel to the longer side of the optical table (item 63) in the field opposite to 'Number of X wells'. As an example, if you are imaging all wells in an ibidi µ-Slide eight-well chamber twice, enter '2' in this field.

283 Enter the number of wells to be imaged in the direction parallel to the shorter side of the optical table in the field opposite to 'Number of Y wells'. As an example, if you are imaging all wells in an ibidi µ-Slide eight-well chamber, enter '2' in this field.

284 Enter the number of FOVs to be imaged in the direction parallel to the longer side of the optical table in the field opposite to 'Number of X FOVs'. As an example, if you are imaging four regions of each well, then enter '4' in this field. Beware that the product of this field and the field opposite from 'Number of Y FOVs' has to equal the number of regions to be imaged in each well.

285 Enter the number of FOVs to be imaged in the direction parallel to the shorter side of the optical table in the field opposite from 'Number of Y FOVs'. As an example, if you are imaging four regions of each well, enter '4' in this field. Beware that the product of this field and the field opposite from 'Number of X FOVs' has to equal the number of regions to be imaged in each well.

286 Enter the laser(s) to be used for imaging each region in the fields opposite from 'Lasers'. Lasers will be switched on according to the sequence in which they are entered. As an example, if you would like to image each region by using the (638 nm) red and then (561 nm) green lasers (items 3 and 7), enter 'Red' in the field opposite from 'Lasers' and 'Green' in the field opposite from 'Red'. Beware that the first letter of each entered laser line has to be upper case.

287 Enter the number of times (i.e., repeats) the mounted sample is to be imaged in the objective.

288 Enter the distance between each well in the direction parallel to the longer side of the optical table in the field opposite from 'X wells distance (um)' in micrometers. As an example, if the distance between each well in the direction parallel to the longer side of the optical table is 4.5 mm, enter '4500' in this field. Beware that if you would like to image at non-periodic locations, the entry in this field will be ignored.

289 Enter the distance between each well in the direction parallel to the shorter side of the optical table in the field opposite from 'Y wells distance (um)' in micrometers. As an example, if the distance between each well in the direction parallel to the shorter side of the optical table is 4.5 mm, enter '4500' in this field. Beware that if you would like to image at non-periodic locations, the entry in this field will be ignored.

290 Enter the distance between each FOV in the direction parallel to the longer side of the optical table in the field opposite from 'X FOVs distance (um)' in micrometers. As an example, if the distance between each well in the direction parallel to the shorter side of the optical table is 0.1 mm, enter '100' in this field.

291 Enter the distance between each FOV in the direction parallel to the shorter side of the optical table in the field opposite from 'Y FOVs distance (um)' in micrometers. As an example, if the distance between each well in the direction parallel to the shorter side of the optical table is 0.1 mm, enter '100' in this field.

292 Enter the number of frames to be imaged for each entered laser in the fields opposite from 'Frames'. Each laser will be switched on for the number of frames entered. As an example, if you would like to image each region by using the (638 nm) red and then (561 nm) green lasers (items 3 and 7), enter '2' in this field.
Stage 23: switching off the microscope  ● Timing 5 min

301 Create a new folder on the desktop and rename it as ‘wished’.

302 Select the ‘Acquisition path’ on the NanoPro 1.0 control software window (Fig. 4 in the visual operation guide) and choose the created folder.

303 Toggle the ‘Sequence switch’ on the NanoPro 1.0 control software window (Fig. 4 in the visual operation guide).

304 Select the ‘Sequence path’ on the NanoPro 1.0 control software window (Fig. 4 in the visual operation guide) and choose the ‘Sequence.csv’ file in the folder unzipped in Step 205.

305 Perform Steps 268–271.

? TROUBLESHOOTING

Stage 23: switching off the microscope

306 Press the ‘Stop’ button on the NanoPro 1.0 control software window (Fig. 4 in the visual operation guide). Close the NanoPro 1.0 control software window.

307 Close and switch off the computer (item 41).

308 Switch off the power button (item 50) on the power strip.

? TROUBLESHOOTING

309 Switch off the main power outlet to which the power strip is connected.

310 Clean the objective from excess oil by using lens-cleaning tissue (item 142) and acetone or ethanol (see https://microscopy.duke.edu/guides/clean-objective).
## Troubleshooting

Troubleshooting advice can be found in Table 4.

| Step | Problem | Possible reason | Solution |
|------|---------|----------------|----------|
| 201, 202 | Some or all of the microscope’s components are not switched on when the main power outlet is switched on | Current is not flowing through the main power outlet | Plug the power strip into another main power outlet. If the problem persists, consult the electronic/electrical workshop of your department/institution to advise on the root cause of the problem and devise an appropriate solution. The power strip is faulty Replace the power strip The switch on one or more of the microscope’s components is not switched on Switch on all of the microscope’s components as described in Step 201 One or more of the microscope’s components are damaged Contact the supplier of the damaged components for advice on the root cause of the problem and to arrange for maintenance or replacement |
| 212 | Supplementary NanoPro 1.0 software (item 138) cannot be opened, giving an error | One or more libraries are not installed | Follow Steps 203–212 carefully to ensure that all libraries are installed and that the folder named ‘dependencies’ contains all the files required by NanoPro 1.0 to operate. The message ‘(Component) is not connected’ is displayed in the ‘[message box]’ of the NanoPro 1.0 control software window (Fig. 4 in the visual operation guide; see Code availability) | Connect the component as described in Steps 164–184 Switch on the component as described in Step 201 Contact the supplier of the damaged component for advice on the root cause of the problem and to arrange for maintenance or replacement. The component is not properly registered in the ‘Configuration.csv’ file (see Step 211) Register the component as described in Step 211 The component is not set up yet Wait a few minutes after switching on the computer before trying to open the NanoPro 1.0 software. If the problem persists, switch off the power strip, wait a few seconds, switch on the power strip, restart the computer and open the NanoPro 1.0 software. The USB hub is not working (item 42) Connect the USB hub to the power strip as described in Step 184 Connect the USB to the computer as described in Step 184 The USB hub is faulty and needs replacement. Replace it with a new hub or any other electrically powered USB 2.0 hub with at least six USB ports |
| 214, 232, 236, 260, 268, 296 and 305 | Laser light is not emitted when the ‘Live’ or ‘Acquire’ buttons are pressed on the control joystick (item 43) (Fig. 6 in the visual operation guide; see Code availability) | The four-position slider is damaged Contact the supplier of the four-position slider for advice on the root cause of the problem and to arrange for maintenance or replacement. The four-position slider is stuck in position Refer to the instruction manual provided by the supplier on moving the four-position slider from the three black buttons on the connected red electronic board before attempting to control the movement of the slider by using the NanoPro 1.0 software. The four-position slider is not connected to the red electronic board Connect the four-position slider to the red electronic board as described in Steps 123 and 175 The red electronic board is not connected to the computer Connect the red electronic board to the computer as described in Step 184 One or more of the lasers are not connected Connect the lasers (items 4–8) to the digital analog converter device (item 44) as described in Steps 165–172, 180 and 181 Ensure that three LEDs on the electronic circuits connected to the ultraviolet (405 nm), blue (488 nm) and red (638 nm) are emitting. If less than three LEDs are emitting, then this would indicate that connections are not properly secured. If more than three LEDs are emitting, then this would indicate that the laser(s) are... |
Table 4 (continued)

| Step       | Problem                                                                 | Possible reason                                                                 | Solution                                                                 |
|------------|-------------------------------------------------------------------------|---------------------------------------------------------------------------------|--------------------------------------------------------------------------|
| 248, 253,  |
| 296 and 305| White light is not emitted from the LED (item 9) when the 'Live' or 'Acquire' | The LED is not connected to the LED driver (item 54)                            | Connect the LED to the LED driver as described in Step 179              |
|            | buttons are pressed on the control joystick                              | The LED driver is not connected to the computer                                  | Connect the LED driver to the computer as described in Steps 182 and 184 |
|            |                                                                         | The LED power is low                                                            | Rotate the knob on the LED driver clockwise to the position labeled 'LIMIT' |
|            |                                                                         | The switch on the LED driver is not in a correct position                      | Flick the switch on the LED driver to the position labeled ‘TRIG’        |
| 248, 254,  |
| 260, 269,  |
| 273 and 305| The image is moving/ vibrating when lightly tapping on the breadboard     | The FOV is brighter on one side (Fig. 4b)                                      | Align the emission path as described in Step 237                        |
|            | (item 64)                                                               | The FOV is cropped (Fig. 4c)                                                    | Align the emission path as described in Step 238                        |
|            |                                                                         | Bursts do not appear as symmetric concentric circles out of focus (Fig. 4d)    | Align the sample holder properly, as described in Steps 199 and 200. Pay close attention to match, as perfectly as possible, the position of the air bubble in the spirit level on the top plate of the microscope box and the sample holder |
|            |                                                                         | The sample holder (item 134) is not aligned parallel to the top plate of the    |                                                                          |
|            |                                                                         | microscope box (item 132)                                                      |                                                                          |
|            |                                                                         | Bursts appear elongated in and out of focus                                    | Ensure that the O-rings (items 143 and 144) are appropriately slotted as described in Step 73 |
|            |                                                                         | The vibration motor (item 61) is rotating at a high speed, causing vibrations   | Increase the rotation speed of the vibration motor by rotating the screw on the variable DC power source (item 47) anti-clockwise by using the slotted screwdriver (item 125) until the maximum limit |
|            |                                                                         | to the sample and microscope                                                   |                                                                          |
|            |                                                                         | The vibration motor (item 61) is rotating at a low speed, causing ineffective   | Connect the vibration motor to the variable DC power source, as described in Step 182 |
|            |                                                                         | elimination of the speckle pattern resulting from the propagation of light in   |                                                                          |
|            |                                                                         | the multimode fiber (item 1)                                                   |                                                                          |
|            |                                                                         | The vibration motor is not working                                             |                                                                          |
|            |                                                                         | The image appears patchy (i.e., with speckles or dark regions; Fig. 4f)        |                                                                          |
|            |                                                                         | Background, not from the sample, overwhelms the FOV                            |                                                                          |
|            |                                                                         | External light sources are switched on                                          | Switch off all light sources, including the room’s main light and any other lamps |
|            |                                                                         | The front plate of the microscope box is stowed away                           | Fix the front plate to the microscope box as described in Steps 243 and 244 |
|            |                                                                         | Emission filters are not properly inserted                                     | Properly insert the emission filters as described in Steps 121 and 122 |
| 249 and 261| Focusing on sample is not possible                                       | The thickness of the coverslip is not appropriate                               | Use #1.5 coverslips only                                                |
|            |                                                                         | Insufficient amount of immersion oil (item 140) on the objective lens          | Squirt one or two drops of the immersion oil on the objective lens       |
| 254, 269,  |
| 273 and 305| The sample is not fixed in position on the sample holder                  | Fix the sample on the sample holder by using the installed clips               |                                                                          |
|            |                                                                         |                                                                                |                                                                          |
Timing

Stage 1, procurement and space preparation: ≤6 months
Stage 2, fabrication: ≤3 months
Stage 3, unpackaging and installation: 2 d
Stage 4, assembling the laser combiner: 3 h
Stage 5, first partial assembly of the microscope box: 1 h
Stage 6, assembling the excitation module and focus-stabilization system: 2 h
Stage 7, second partial assembly of the microscope box: 1 h
Stage 8, assembling the emission module: 1 h
Stage 9, assembling the sample stage: 2 h
Stage 10, setting up electrical connections: 2 h
Stage 11, assembling the laser-combiner enclosure: 30 min
Stage 12, assembling the sample enclosure: 2 h
Stage 13, assembling and aligning the sample holder: 30 min
Stage 14, switching on the microscope: 5 min
Stage 15, setting up the computer and installing the NanoPro 1.0 control software: 2 h
Stage 16, aligning the laser combiner: 4 h
Stage 17, aligning the excitation and emission paths: 20 min
Stage 18, aligning the focus-stabilization system: 30 min

Table 4 (continued)

| Step          | Problem                                                                 | Possible reason                                                                 | Solution                                                                                          |
|---------------|-------------------------------------------------------------------------|--------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|
| 262, 273 and 298 | The ['Focus status box'] does not display 'Focused' even when the sample is focused | The thickness of the coverslip is not appropriate | Use #1.5 coverslips only                                                                            |
|               | The thickness of the coverslip is not appropriate                       | The laser power is low                                                         | Increase the laser power by using the control joystick                                           |
|               |                                                                         | Incorrect laser line in use                                                    | Switch on the laser line that matches the excitation spectrum of the fluorescent sample         |
|               |                                                                         | The four-position slider has not moved position to the right emission filter  | Refer to the problem with the four-position slider (above) for causes and solutions             |
|               |                                                                         | The sample is not properly fluorescently labeled                              | Ensure that the sample is appropriately stained                                                  |
|               |                                                                         | The thickness of the coverslip is not appropriate                              | Use #1.5 coverslips only                                                                            |
|               |                                                                         | Insufficient amount of immersion oil (item 140) on the objective lens          | Squirt one or two drops of the immersion oil on the objective lens                                |
|               |                                                                         | Lasers(s) are misaligned. The sample is not illuminated in TIRF               | Align the lasers appropriately as described in Steps 213-239                                      |
|               |                                                                         | The autofocus system is not aligned                                            | Align the focus-stabilization system as described in Steps 240-245                                |
|               |                                                                         | The compact diode laser is not switched on or emitting                        | Switch on the compact diode laser as described in Step 201                                        |
|               |                                                                         | The compact diode laser is damaged                                             | Contact the supplier of the compact diode laser for advice on the root cause of the problem and to arrange for maintenance or replacement |
|               |                                                                         | The position aligner is not connected                                          | Connect the position aligner as described in Steps 173 and 184                                     |
|               |                                                                         | The position aligner is not switched on                                         | Contact the supplier of the position aligner for advice on the root cause of the problem and to arrange for maintenance or replacement |
| 308           | Power trips when the power strip (item 50) is switched on or off        | The power strip is faulty Current overload                                     | Replace the power strip                                                                           |
|               |                                                                         |                                                                                  | Switch off power from the wall socket first, rather than the power strip. If the problem persists, consult the electronic/electrical workshop of your department/institution to advise on the root cause of the problem and devise an appropriate solution |

NATURE PROTOCOLS PROTOCOL

2615
Fig. 5 | Exemplary images of cellular and recombinant macromolecular complexes obtained by using NanoPro 1.0. **a**, Microtubules immunostained with a primary/secondary antibody system and imaged by using dSTORM. **b**, Clathrin pits immunostained with a primary/secondary antibody system and imaged by using dSTORM. **c**, Multi-target imaging of microtubules and clathrin pits by using exchange DNA-PAINT. **d**, Multi-sample imaging of recombinant alpha-synuclein aggregates by using aptamer-based DNA-PAINT at different concentrations: (1) 2.8 µM, (2) 1.4 µM, (3) 700 nM, (4) 350 nM, (5) 175 nM, (6) 87.5 nM, (7) 43.75 nM and (8) 21.875 nM. Scale bars: **a–c**, 10 µm; **d**, 1 µm. Preparation protocols can be found in the Supplementary Information.
Stage 19, measuring the camera pixel size: 30 min
Stage 20, imaging ground-truth samples to establish performance: 1 h
Stage 21, data processing: 1 h
Stage 22, multi-sample acquisitions: dependent on the sequence of events
Stage 23, switching off the microscope: 5 min

Anticipated results

The assembly of NanoPro 1.0 is a relatively long process. Despite the extensive documentation of the process and accompanying illustrations, it is expected that the microscope would initially turn out to be misaligned (Fig. 4, a–d), dysfunctional, or incapable of producing high-quality images (Fig. 4, e–f). All these problems, their causes and solutions are summarized in the Troubleshooting section.

When well aligned and operational, the NanoPro 1.0 will be capable of acquiring high-resolution (20-nm) images of ground-truth nanorulers (Fig. 3), single- and multi-target cellular structures by using dSTORM and DNA-PAINT with different fluorophores (Fig. 5, a–c; Supplementary Note 1) and multiple samples (Fig. 5d; Supplementary Note 1) without user intervention and from 8 and up to 50 samples (dependent on the type of multi-well chamber used).

Imaging cellular or recombinant structures by using the different SMLM techniques requires extensive expertise in sample preparation and data processing. Here, we performed imaging of these structures without significant optimization or the use of advanced image-processing algorithms that could improve the representation of the underlying biological structures and push the resolution below 10 nm. Users of the NanoPro 1.0 must optimize these factors depending on their imaging requirements.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

Updated versions of the source code for NanoPro 1.0, as well as guiding instructions (visual assembly, alignment and operation guides and instructional videos), can be obtained from https://github.com/jdanial/NanoPro and are archived in Zenodo. A compilation of NanoPro 1.0 for the Windows operating system is available in the GitHub repository.

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Author contributions
J.S.H.D. and D.K. conceived and designed the study. M.W. and J.S.H.D. designed the microscope. J.S.H.D., J.Y.L.L. and Y.W. assembled the microscope. J.S.H.D. wrote the NanoPro 1.0 software with input from J.Y.L.L. and Y.W. J.S.H.D., J.Y.L.L. and Y.W. performed the analysis. E.D. prepared all cellular samples. M.R.C., D.E., J.Y.L.L., Y.W. and J.S.H.D. revised the protocol. J.S.H.D. wrote the manuscript with input from all authors.

Competing interests
The authors declare no competing interests.

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