Supplemental Document

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Self-contained and modular structured illumination microscope: supplement

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Self-contained and modular structured illumination microscope
Supplementary Information

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1 HIT-SIM optics

A list of HIT-SIM optics, accompanying calculations, and code and settings files are available online (https://doi.org/10.5281/zenodo.4557034). The layout and hardware can be customized following the guidelines provided.

Supplementary figure 1 provides the rationale for using two identical dichroic mirrors in the instrument. These compensate for the phase shifts that occur between the reflection of *p*- and *s*-polarized light on the dichroic surface, which would otherwise lead to a low modulation depth of the SIM pattern.

2 Alignment scheme

The following instructions provide a high-level overview of the steps required to build and align the instrument. We used a non scientific CMOS camera (IDS µEye UI-3060CP-M-GL Rev.2) in the alignment of the SIM module. A diagram of the optical layout is shown in supplementary figure 2.

2.1 Phase 1: the skeleton

1. Designate room for the excitation, body and emission sections.
2. Define the path of the laser you will use (before entering the SIM module; supplementary figure 3), by placing two irises.
3. Mount the SLM on its separate breadboard and the correct height (i.e., matching the optical axis of the dovetail)
4. Align the SLM to 2 irises on the dovetail (use a low power alignment laser that aligns trough the path that you will use for the actual laser – 2 irises and 2 silver mirrors, into the SIM module).
5. Use a silver mirror to hit L3 straight (i.e., directionality of the beam does not change with/without L3).
6. Adjust height of the stage/body or SIM module to roughly hit the center of the lens (this step is skipped when using the parts of the parts list).

7. Construct the microscope body (4 Thorlabs 30 mm cage cubes) and align both SIM dichroics to exit the microscope centered and straight (i.e., hitting the ceiling trough 2 irises). **Of note:** The body is built up as depicted in figure 2 of the main text, and uses Thorlabs “ER" rods to link all adjacent cubes together.

8. XY position L3 to hit it centered and straight.

2.1.1 Phase 2: The Detection

1. Bring the camera used for actual imaging into infinity focus with the use of L4.

   Option 1: position the camera to look at an object far away (> 100 m), and bring it into focus with L4 (for convenience, we use Thorlabs lens tubes to hold this lens, so that the distance between lens and camera does not change when moving the camera back to the setup).
   Option 2: position the camera to look at an object that is far in the room (> 3 m), and bring it into focus with L4. Use the thin lens formula $\frac{1}{d_{\text{object}}} + \frac{1}{f+\text{offset}} = \frac{1}{f}$, to calculate the acquired offset to image an object at infinity.

2. Attach the camera + L4 to the body (this is the emission path).

3. Place the objective into the body and adjust it to touch the sample. Get it into focus (use transmission light and the camera for imaging).

4. Center the camera on the transmission image by using the mirror in front of L4.

5. Illuminate (alignment laser) the objective via the SLM, without sample, and position the L3 to collimate the beam.

   Option 1: use a shear plate/shearing interferometer to achieve collimated light.

   Option 2: look to the spot on the ceiling and position it to achieve a collimated beam (non-diverging/converging).

2.1.2 Phase 3: The microscope

1. Make a ‘Fixed Locally Observed, Far away Image’ (FLOFI) with the first SIM module lens (f = 400 mm) and the SLM as described below (Setting up a FLOFI). This is done so that the SLM mimics an object located very far away.

2. Replace the objective with the IDS camera (+ lens); by using lens tubes you can simply screw this in; and position the 140 mm lens to have the SLM in focus on the camera. L3 is kept in the system during this procedure. **Of note:** Make sure you still go through the center of the dovetail. This can be done by minor adjustments with the SLM and the silver mirror in front of the SLM.

3. Remove L3 and place a piece of aluminum foil or a thin object that has structure (predecessor of the OSA) between the 400 mm and 140 mm lenses. Now move the OSA so that the configuration OSA // 140 mm lens // 50 mm lens + camera gives an image of the OSA on the camera. With this step the OSA’s ideal position is determined. In order to position the 400 mm lens correct, now place the 50 mm lens + camera between the SLM and 400 mm lens (directed towards the 400 mm lens) and move the 400 mm lens to image the OSA. The 400 mm and 140 mm lenses are now in 4f. Alternatively, you could bypass the SLM and use a laser and shear plate, if this is practically possible. **Of note:** Make sure you still go through the center of the dovetail. This can be done by minor adjustments with the SLM and the silver mirror in front of the SLM.

4. Now, make the initial FLOFI again by positioning the camera + lens in front of the 400 mm lens and SLM, and move the SLM to be in focus. **Of note:** If you are making use of a breadboard for the SIM module, the SLM might need to be positioned off of the breadboard. Since this would not be stable, simply move the entire breadboard further from the body and realign the SIM module again. This will shift the position of the optics on the breadboard.

5. From this point the imaging laser needs to be used (**Of note:** modulate the laser to a low power as a safety measure). Realign the tilt of the SLM and mirror preceding it, to align the laser with the optical axis above the dovetail and re-adjust the XY position of L2 to hit L3 centered and straight.

6. Make sure the laser goes straight through L3, by extending the body with rods and an iris in front/after the lens.

7. Now place the OSA in the imaging plane in between the 400 and 140 mm lenses.

8. Select the orders that are desired (start off with aluminum foil, through which holes are poked). **Of note:** You could use a marker and bleach away the points of interest, before poking holes.

9. Place the QWP in between the OSA and 400 mm lens.

10. Place the PP in between the OSA and the 140 mm lens.

Setting up a FLOFI

1. Position a lens (e.g., f = 50 mm) in front of a small camera (e.g., an IDS camera), and make sure the camera is infinity focused with this lens (i.e., focus at a church or far away object).

2. Position this camera + lens so that it is in the direction of the 400 mm lens and the SLM. - configuration: IDS + lens // 400 lens // SLM

3. Move the 400 mm lens so that the SLM comes into focus on the small camera. (The SLM is now at optical 'infinity'.)

2.1.3 General remarks

- SIM can be done without polarization optics, but for the best quality it is highly recommended to use the polarization optics (2 SIM dichroics, PP and QWP).
- The OSA can initially be made out of aluminum foil, mounted in a cage plate. For the best result, we propose to use a camera and define the exact positions of the spots, followed by a machined mask which is positioned correctly with an XY mount.
- The SLM gives a wide spread of points, which will illuminate the entire room if not enclosed. We suggest to provide housing for the SIM module, and if possible for the entire setup. The latter is not only useful for light leaking out of the system, but also for preventing light to enter, dust to accumulate or gusts of air causing vibrations.

3 SLM pattern selection

Download either the original version of the grating search algorithm[1] for Matlab or the multi-color version[2]. Select a set of patterns that give 6 first order spots that are easy to filter out from any spurious spots that are present. The resulting set can be uploaded into the SLM software (Metrocon by ForthDD) and configured by using a repertoire. Hardware activation needs to be selected, to allow control via the Arduino. Likewise, the checkbox for FINISH signal should be ticked, so that the pattern sequence is reset when imaging is stopped. Multi-color acquisitions require that the individual patterns for each color are added in a consecutive sequence, which also defines the order in which the different colors must be acquired. Our own SLM repertoire is included in these supplementary documents (.repz extension). This repertoire can only be used for the SLM (SXGA-3DM) used in this paper. Using a different SLM also means developing a new repertoire.

[1]https://github.com/nanoimaging/fastSIM_GratingSearchforSLM
[2]https://github.com/fairSIM/fastSIM-GratingSearch

4 Electronics

The microscope control is maintained via an Arduino Uno (supplementary figure 4 and supplementary figure 5 show the arduino wiring diagram and electrical connections respectively) and can be downloaded from https://github.com/fairSIM/arduino-flcos-code. It uses digital signals, i.e., **high** and **low** logic levels, to synchronize the laser light source, camera and SLM. While tailored to the specific SLM, it should be compatible with a wide range of cameras and light sources, as long as they fulfill the following requirements:

The **camera** has to provide an exposure output signal that is active whenever the full region of interest (ROI, subset of pixels selected for imaging) is simultaneously light sensitive for the current frame. On rolling shutter cameras, this signal is often distinct from e.g., a signal showing the exposure of a specific line or any line in the selected ROI. To run without modification, our code additionally expects a gap of 2.5 ms or more between those exposure signals, which on a rolling shutter camera is introduced by the rolling readout, but on a global shutter camera would have to be set manually.

The **laser light source** needs to offer a modulation input that allows to quickly toggle the laser on and off, with a rise/fall time of ideally 10 µs or less. Most pure diode laser sources are easily fast enough, while DPSS lasers might need external modulation, for example by means of an AOTF. If no digital modulation input is available, analog inputs can be used, provided voltage levels match, the modulation is fast enough, and maximum power levels can be controlled in software.

With the camera exposure output and laser modulation input in place, our micro-controller code works as follows to synchronize the components:

a **Waiting for exposure**: In idle state, the µController waits for the camera exposure to become active, i.e., for *camera exposure* to transition to **high**. Additionally, if the internal *needs_reset* flag is set (see point 'd', reset timer, below), and there is no exposure for more than approx. 500 ms, the code jumps into the reset routine.

b **Exposure loop**: Once the camera signals exposure, the micro-controller enters the following loop, during which it continuously monitors the *camera exposure* signal and exits the loop once it transitions back to **low**.

1 The controller triggers the SLM (pulsing the *SLM trigger* line **high** for approximately 10 µs).

2 The SLM now takes 369 µs to load the current SIM pattern and orient all its pixels. It signals completion by pulling *SLM LED enable* to **high**.

3 The micro-controller monitors *SLM LED enable*. As it becomes high, it turns on the laser (*laser enable* goes high).

4 After displaying the image for a set time (determined by the sequence loaded, typically set to 1 ms or 2 ms), the SLM needs to invert its pixels. It request the light source to turn off by dropping *SLM LED enable* to **low**.

5 The micro-controller monitors *SLM LED enable*. As it becomes low, it turns off the laser (*laser enable* goes low).

6 The SLM needs 434 µs to invert its image. Since both the positive and the inverted image can be used in 2-beam SIM, both are set to output an *SLM LED enable* enable signal. After the first, positive image, the micro-controller jumps to '3' here, waiting for the negative image. After the second, negative image, it jumps to '1', to re-trigger the full display sequence.

Note that the displayed SIM pattern (angle and phase) does not change while in the loop, and the loop is integrating exposure and illumination time as long as the camera's exposure signal stays active.

c **Pattern transition**. When *camera exposure* transitions back to **low**, the micro-controller switches of the laser (*laser enable* goes low), and transitions the SLM to the next SIM pattern (by pulsing the *SLM finish* line **high** for approximately 10 µs). In the SLM programming (so-called 'running order'), this signal exists an internal inner loop that repeated the same SIM pattern, and thus activates the next SIM pattern in line.

d **Position unknown flag**. The micro-controller sets a flag marking the SIM pattern position as unknown (*'needs_reset'*), as it keeps no internal count of the position (angle/phase), and the user might have stopped the acquisition at an arbitrary position. The code jumps to 'a', waiting for the next exposure (or the reset timer to trigger and jump into the reset routine).

Reset routine: The reset timer routine (see 'a' and 'd' in the loop above) is triggered whenever there was no exposure for a set amount of time (approximately 500 ms with current settings), and the SIM pattern position on the SLM is not already known to be set to the first position.

This assumes the typical behavior of a user searching for structures on a sample in free-running imaging mode, and stopping after an arbitrary number of image acquisitions. As we do not want to impose restrictions on the acquisition software ("always acquire multiples of 9 images"), we need to reset the SLM to the first SIM pattern after such acquisitions.

The reset procedure loops over sending an *SLM trigger* pulse, waiting 2 ms and sending an *SLM finish* pulse, which each time advanced to the next SIM pattern. The last pattern in sequence is programmed to pull *SPO* to **low**, which breaks the loop and lets the micro-controller know the reset is complete.

It clears the *needs_reset* flag and return to 'a' in the main loop.

The reset delay should be chosen long enough so jitter in camera readout, system control and similar effects do not trigger it inadvertently, but short enough to occur quickly when manually operating the microscope. Once the reset is triggered, it should take less than 50 ms to complete. In automated microscopes, care needs to be takes so that stage movements are either quick enough to not trigger the reset, or slow enough for the reset to complete before the next set of SIM images is taken. The delay is easily adjustable in the micro-controller code.

Debug output: In regular operation, no data connection from the micro-controller to a computer is needed. Two LEDs provide rudimentary feedback, signaling imaging operation ('on' when waiting for exposure, 'off' while running the loop) and SIM pattern reset ('on' while the reset routine is run). Additionally, debug output is available by connecting to the serial port provided by the micro-controller, where (for speed reasons) a character is output for (roughly) every step mentioned in the loops above. For details, please refer to the micro-controller source code.

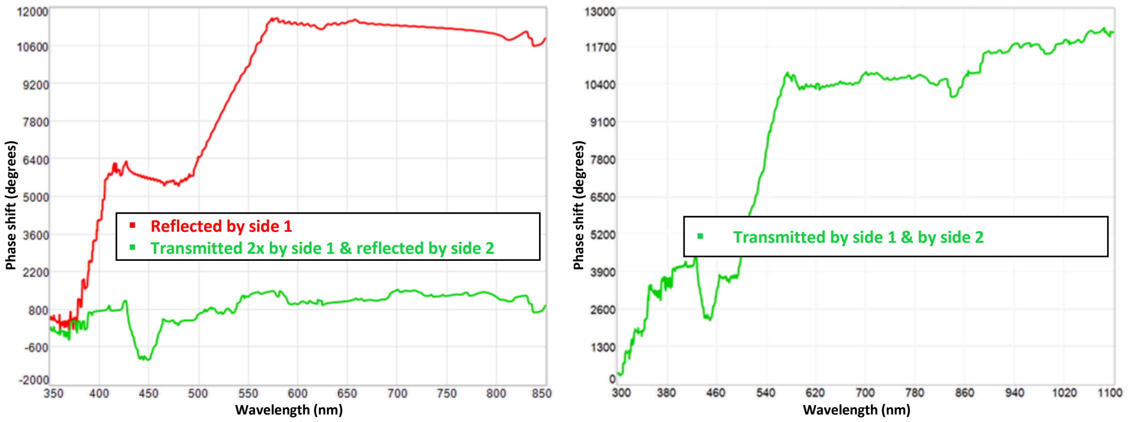
While full debugging of the signal flow requires a logic analyzer or a 4-channel digital storage oscilloscope (DSO), this build-in feedback can often help to diagnose configuration errors such as misconfigured camera output signals without additional hardware.

Multi-color acquisitions require a separate Arduino output pin for each excitation wavelength. The code is then extended so that the different Arduino triggers the first wavelength during the first nine exposures, the second wavelength during the next nine exposures, etc. The entire sequence must also be reset whenever the SLM sequence is reset.

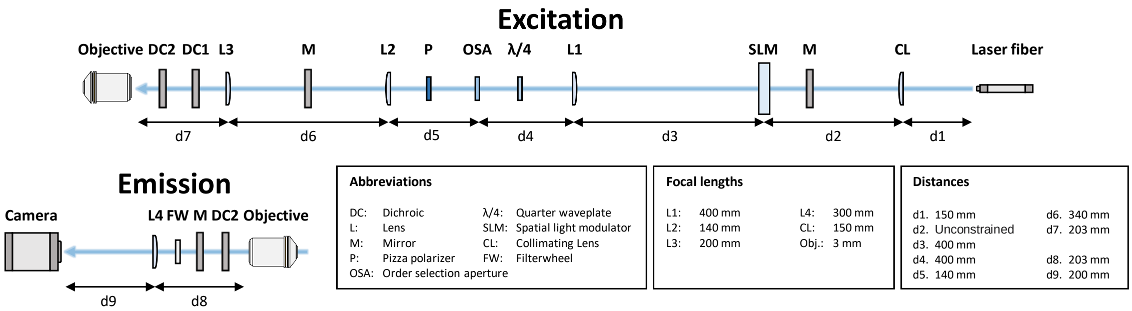
5 Parts list

The list of parts (optics, optomechanics, electronics, ...) is available in the accompanying documents.

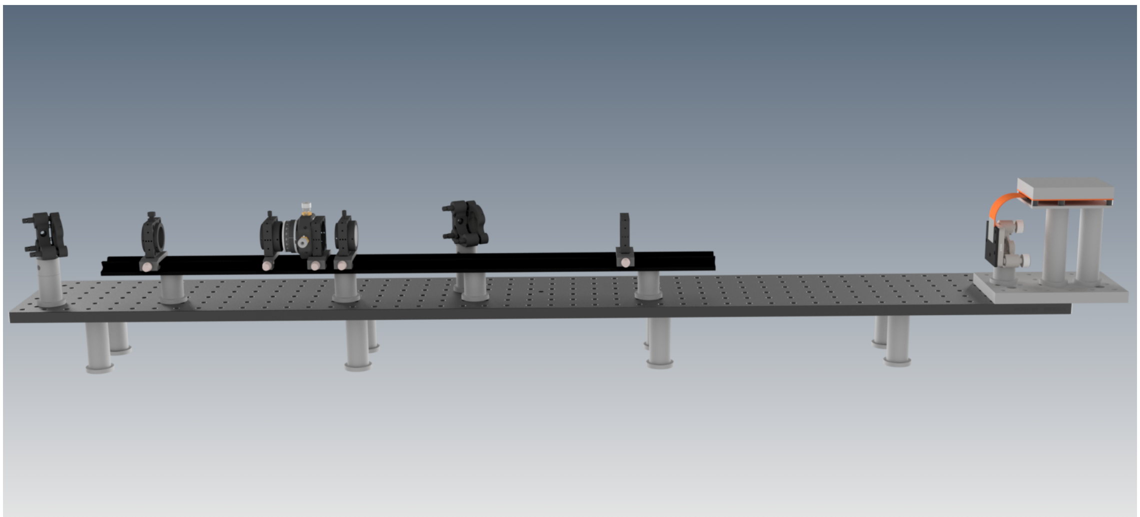
6 Supplementary figures



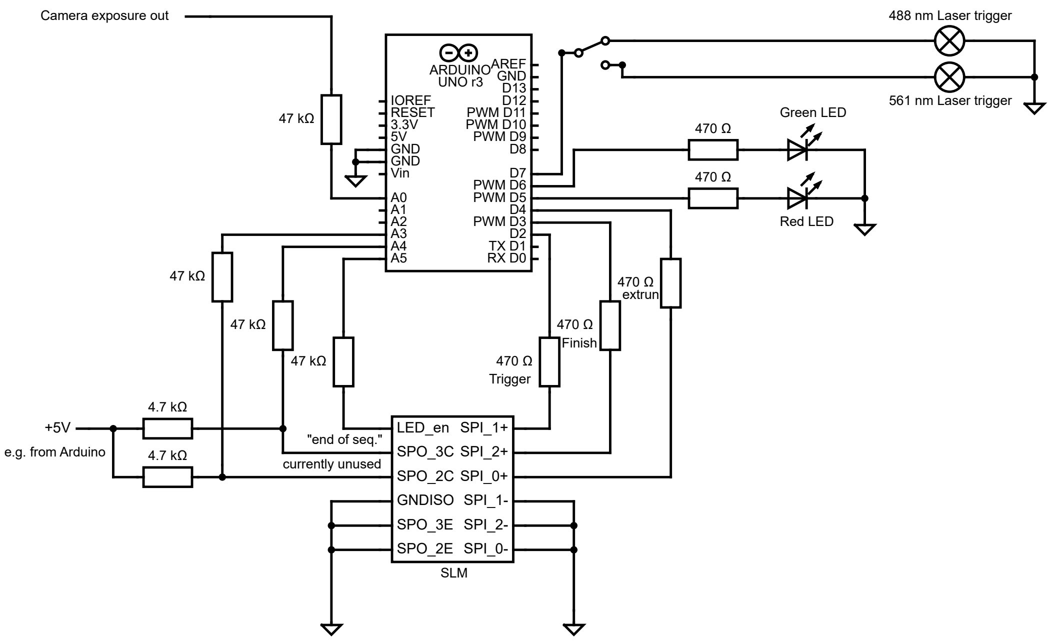
Supplementary Figure 1: P-S polarisation shifts using a single 4 band (Quadband Laser Beamsplitter R405/488/561/635) dichroic. “P-S” in the graph refers to the numerical difference in phase, experienced by p- and s-polarized light reflected or transmitted through the coatings (side 1 and/or 2) of the filter. Left: Reflected light in a dichroic takes two paths; reflecting back off the first side (red), and transmitting through the first side to be reflected off the second side (green). Right: Fully transmitted light also results in a shift. (Image courtesy of Idexcorp.)



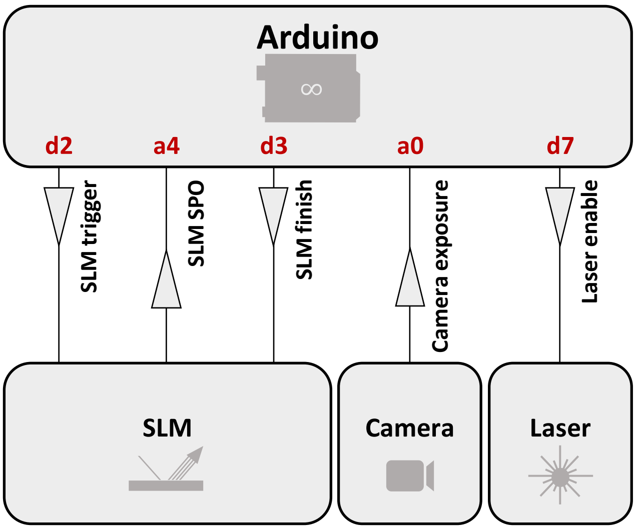
Supplementary Figure 2: Optical diagram of the system, giving approximate distances, focal lengths, and lens orientations. The final alignment should be performed following the detailed alignment scheme provided here.



Supplementary Figure 3: CAD rendering of the SIM module.

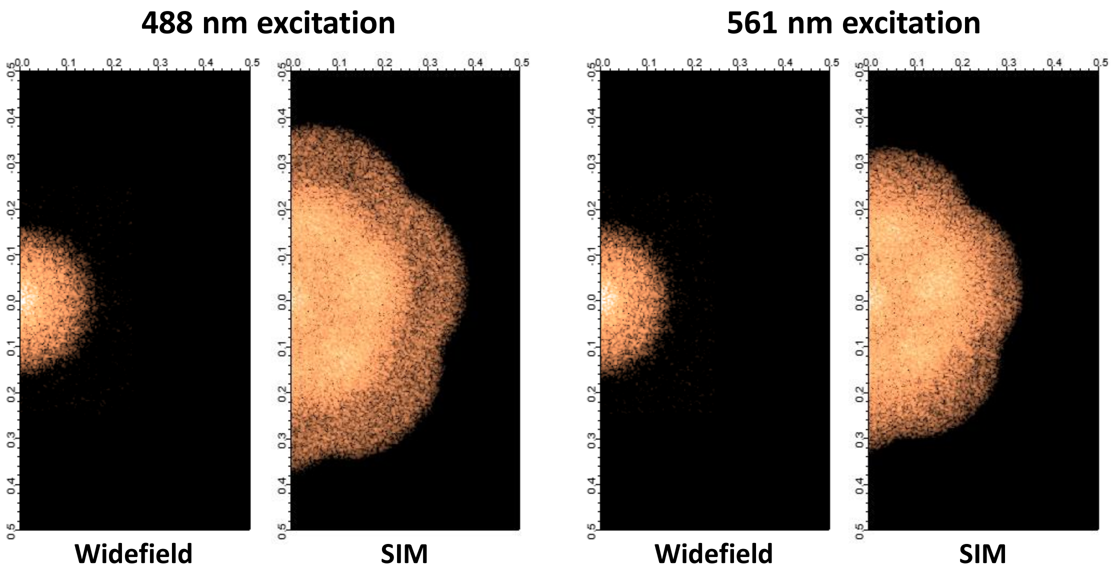


Supplementary Figure 4: Microcontroller wiring diagram. Port D (digital pins d0 to d7) is used as output, port C (labelled ‘analog pins’ by Arduino) as input, see also the Arduino source code. Pins d2, d3 and d4 control the SLM ‘trigger’, ‘finish’ and ‘external run’ (currently not in use) signals. As the SLM inputs use optocouplers, current limiting resistors (see SLM datasheet) have to be used. Pins d5 and d6 power two control leds that signal ready and ‘SLM reset’. Pin d7 controls the laser light source, which directly accepts a TTL-style 5V signal. On the input side, pin a0 reads in the ‘camera exposure’ signal. The 3.3V signal provided by the camera is compatible with the Arduino input. Pins a3, a4 and a5 read the SLM ‘LED enable’, ‘SPO3’ and ‘SPO2’ signal. For the LED signal, the SLM provides a 5V output. For the SPO3 output (signalling the last image in the sequence, used to reset the SLM to this position) and the SPO2 output (currently unused) the SLM offers ‘open collector’ outputs, which have to be provided with a voltage (here 5V, taken from the Arduino) through pull-up resistors (here 4.7 kOhm). The 47 kOhm resistors in line with the Arduino input pins are not necessary for regular function, but offer some protection against misconfiguration, e.g. avoid shortcuts when accidentally setting the pins as outputs in the software. Switching between different light sources is implemented as a physical switch on our system.

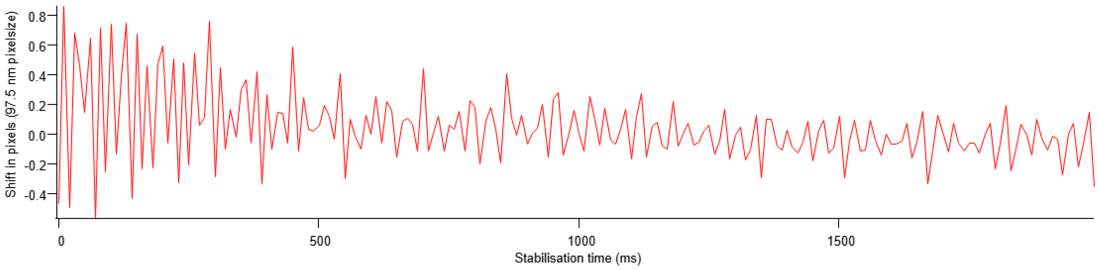


d2: OUT: SLM trigger
d3: OUT: SLM finish
d5: OUT: LED (showing 'SLM is being reset') – not shown
d6: OUT: LED (showing 'waiting for exposure') – not shown
d7: OUT: laser
a0: IN: camera exposure
a4: IN: SLM SPO (on when the SLM is on the last image in the pattern)
a5: IN: SLM LED enable – not shown

Supplementary Figure 5: Scheme of the electrical connections from and to the Arduino Uno. a: analog pins. d: digital pins.



Supplementary Figure 6: OTF of the WF and SIM reconstructed data from main text figure 4. The axes show spatial frequency (per pixel).



Supplementary Figure 7: Stage stability after moving to a set position along the *x* direction.