Chapter 10

Multi-exposure Speckle Imaging for Quantitative Evaluation of Cortical Blood Flow

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Abstract

Laser speckle contrast imaging (LSCI) is a label-free optical imaging technique that can quantify flow dynamics across an entire image. Multi-exposure speckle imaging (MESI) is an extension of LSCI that allows for reproducible and quantifiable measurements of flow. MESI has the potential to provide quantitative cerebral blood flow information in both preclinical and clinical applications; in fact, MESI can be extended to resolve the flow dynamics in any exposed tissue. A MESI system can be divided into three primary components: (i) the illumination optics, consisting of the optical source and a method of modulating and gating the illumination intensity; (ii) the collection optics, consisting of a high-speed camera that can be triggered and gated to match the pulsed illumination; and finally (iii) post-processing hardware and software to extract the flow information from the recorded raw intensity images. In the following protocol, we offer a guide to design, operate, and test a MESI system.

Key words Cerebral blood flow, Optical imaging, Laser speckle contrast imaging, Multi-exposure speckle imaging, Neurosurgery, Intraoperative imaging

1 Introduction

Monitoring cerebral blood flow (CBF) is crucial to the success of neurosurgical interventions and neuroscience applications [1–3]. Currently, imaging techniques such as indocyanine green angiography (ICGA) and digital subtraction angiography (DSA) are used to monitor CBF intraoperatively; however, these techniques suffer from requiring contrast agents, a disruption to a surgical procedure if used intraoperatively, and require radiation exposure in the case of DSA [4, 5].

Laser speckle contrast imaging (LSCI) is a full-field, label-free, optical imaging technique that can provide continuous maps of blood flow; thus, it can be used in an extensive number of applications across neuroscience, dermatology, dentistry, and ophthalmology [6, 7]. Additionally, LSCI has been shown to be a powerful research tool for preclinical stroke studies, primarily with rodent

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models, helping to inform neurosurgical interventions and broadening neuroscience advances. These studies have covered research topics such as monitoring CBF during ischemic stroke induction [8], chronic monitoring of the vasculature remodeling after stroke induction [9–11], investigating the effects of isoflurane-based vasodilation [12], and studying the impact of obesity on CBF [13]. There have also been advancements in research to integrate speckle contrast measurements within a surgical microscope for intraoperative use [1, 14].

Laser speckle is the random interference pattern produced when coherent light scatters from a rough surface or inhomogeneous medium. When a sample contains scattering particles in motion, the speckle will vary temporally, creating a speckle pattern. When integrated over the exposure time of the camera, this speckle pattern will encode information about the underlying particle motion. Thus, a measure of flow can be obtained by quantifying the spatial or temporal statistics of the speckle pattern. This speckle pattern requires calculating the local speckle contrast, K, defined as

$$K = \frac{\sigma}{\langle I \rangle}, \tag{1}$$

where σ is the standard deviation and $\langle I \rangle$ is the average intensity over a sliding window of pixels (e.g., 7×7) of the imaged data.

These speckle contrast values are only indicative of the amount of motion in a sample and are not proportional to particle speed or volumetric flow. To determine a measure of flow in the sample, it is necessary to relate the speckle contrast value to the correlation time constant (τ_c) of the speckle autocorrelation function, which is inversely related to the speed of the scatters, although the exact nature of this relationship varies with the portion of the vasculature sampled by the detected light [15]. That is, the inverse correlation time (ICT), defined as $1/\tau_c$, is related to the flow of scatters (in this application the flow of red blood cells). The relationship between speckle contrast and ICT can be defined as follows:

$$K(T, \tau_c)^2 = \beta\left(\frac{e^{-2x} - 1 + 2x}{2x^2}\right), \tag{2}$$

where $x = T/\tau_c$, β is a constant instrumentation factor, T is the exposure time of the camera, and τ_c is the correlation time constant.

However, LSCI suffers from several drawbacks, namely, a high dependency upon instrumentation, it does not account for the effect of static scatterers that are present in actual tissue, and it does not account for noise [16]. Due to these limitations, LSCI is typically limited to measurements of the relative changes in blood flow within a single subject during a single experiment, and it is challenging to provide consistent, reproducible, quantitative measures of flow.

Multi-exposure speckle imaging (MESI) was developed as an extension of LSCI to address these issues. The primary goal of MESI is to provide a more robust estimate of the correlation time constant (τ_c) that is repeatable and quantifiable. MESI requires collecting LSCI images over a wide range of camera exposure times to properly sample the underlying flow dynamics. In this protocol, and in many of our lab's experiments, 15 MESI images spanning three decades of exposure times (50 μs–80 ms) are chosen [10, 12, 15, 16]; however, it is not mandatory, and in fact, some published work from our lab has investigated using a select fewer number of exposure times [17]. While the complexity of the MESI hardware has been challenging to integrate into a clinical setting [1], MESI has been used for a wide array of in vivo mouse studies.

MESI is based upon a more rigorous dynamic light scattering model that accounts for static scattering events and non-ideal conditions, producing the MESI equation [12]

$$K(T,\tau_c)^2 = \beta\left[\rho^2\frac{e^{-2x}-1+2x}{2x^2}+4\rho(1-\rho)\frac{e^{-x}-1+x}{x^2}+\nu\right] \tag{3}$$

where $x = T/\tau_c$, ρ is the ratio of intensity undergoing dynamic scattering events to the total intensity, ν is the noise arising from experimental noise and due to simplifying assumptions made in the model, β is a constant instrumentation factor, T is the camera exposure time, and τ_c is the correlation time constant. By collecting this sequence of images at various values of T, it is possible to fully resolve the shape of K^2 and properly solve for τ_c. Solving for τ_c from the experimental speckle contrast values requires implementing a fitting procedure to solve for β, ρ, ν, and τ_c in Eq. 3. This process is done on a pixel-by-pixel basis. Thus, MESI can create flow maps over the entire region of interest (ROI) that is being imaged.

A typical MESI imaging system involves three major components: illumination optics, image acquisition and the collection optics, and post-processing tools. The illumination optics include both optical and electrical components. A stable laser source, that can be directed toward the sample under test, and a fast, triggerable camera are required. Furthermore, MESI requires precise control over both the camera exposure time and the laser intensity. Varying the exposure time alone would result in shot noise overwhelming the speckle signal at longer exposure times because of differences in intensity. However, by modulating the illuminating laser light, the average intensity of the images can be held constant. Optical devices such as acousto-optic modulators (AOMs) have historically been used for precise control over laser intensity and duration, but manual laser diode modulation via the laser controller is also possible. A multi-function I/O device that can communicate with a

computer, the AOM, and the camera is required for these control electronics.

The procedure in this protocol can be summarized as follows: first, set up and test the optical and electrical components, respectively. Then ensure that the sample under test is properly illuminated by verifying the laser alignment. A MESI calibration is required prior to the MESI acquisition phase. Finally, once the raw images have been recorded, the data must be processed by first computing the speckle contrast images and finally creating the ICT maps that provide quantitative flow information.

2 Materials

2.1 Illumination Optics

1. Illumination source: A wavelength-stabilized laser diode (ideally in the 600–850 nm wavelength range), capable of generating a decent amount of power to ensure sufficient light at the shortest exposure time, is the ideal choice of illumination for MESI (e.g., the 300 mW LD785-SEV300 from Thorlabs, Inc.). If a bare diode laser is used, **items 2–4** are required, but if a complete laser diode system is used, **items 2–4** are not required.
2. Temperature-controlled housing: Mount the laser diode in a laser diode mount with an integrated temperature controller.
3. Temperature controller: For stability and repeatability, use a temperature controller (TEC) to set and maintain the laser diode operating temperature.
4. Laser diode controller: For the stability and repeatability of the illumination light, drive the laser diode with a constant current, controlled by a laser diode controller (LDC) that interacts with the laser diode mounting.
5. Isolation: Use a free space optical isolator to minimize back reflections that interfere with single frequency performance.
6. Fiber patch cable (optional): Couple the emitted light into a fiber-optic cable to correct any beam shape irregularities (*see* **Note 1**).
7. Light modulation: Use a free space AOM (e.g., AOMO 3100-125, Gooch and Housego) and an RF driver (97-03307-34, Gooch and Housego) to modulate the intensity of the collimated laser light, ensuring the successful implementation of MESI. Use an iris to isolate the first-order diffracted light from the free space AOM.
8. Control electronics: Use a multifunction I/O device (USB-6363, National Instruments Corp.), referred to as the data acquisition hardware (DAQ), to produce the camera exposure trigger signals and AOM modulation voltages. The drivers

and associated libraries (NI-DAQmx library) for the DAQ will need to be installed.

9. Mirrors: Use steering mirrors as needed throughout the system to direct light, ensure maximum power output, and proper sample illumination (*see* **Note 2**).
10. Power: Use a DC power supply to power the RF driver.

2.2 Image Acquisition

1. Collection optics: Install a pair of camera lenses to image the scattered light and direct the light to the camera sensor. A common configuration is to couple two camera lenses (e.g., 50 mm Nikon DSLR lenses) face to face and set their foci to infinity in a macro-lens arrangement.
2. Filter: Place a bandpass filter, centered around your laser operating wavelength (785 nm in our system), along the collection optics path to remove any background noise (white light) from the collected light.
3. Camera: Use a high-speed CMOS camera that can be triggered from an external source (such as the acA1920-155um Basler AG). The camera can be monochromatic as only pixel intensity is required.
4. Image collection software: Install camera software that allows for image collection, for example, the pylon Camera Software Suite for Basler cameras (*see* **Note 3**).
5. Light intensity calibration: Determine the control the voltages supplied to the AOM so that light intensity levels are balanced at each exposure time (*see* **Note 4**). The voltage for each exposure duration should be recorded.
6. Camera exposure time gating: Define the chosen exposure times for MESI. These exposure times will be needed by the DAQ, the image collection software, and the host computer to properly gate the camera and modulate the AOM to the appropriate exposure times (*see* **Note 4** for details about the gating process, and *see* **Note 5** about the choice of camera exposure times).

2.3 Post-processing

1. Computing power: Use a workstation (desktop or laptop) with ample storage and strong computing capabilities. Speckle imaging records large amounts of data and requires fast processing to quickly produce flow images.
2. Processing software: While many signal and image processing and computing platforms exist and can be used, the general approach to solve the MESI equation is via nonlinear least squares curve fitting.

3 Methods

3.1 System Setup

For ease of testing and design, it is highly suggested to first build the system on an optical table or optical breadboard and test with controllable microfluidic channels or flow phantoms, prior to attempting in vivo imaging. Additionally, follow all laser safety guidelines that apply for the specific class of the laser chosen (proper PPE and signage).

3.1.1 Optical Components

1. Use Fig. 1 as a reference schematic for the layout and connectivity of the optical components.
2. Connect the temperature controller (TEC) and the laser diode controller (LDC) to the mounted laser diode. Operate the LDC at the suggested operating current from the laser diode data sheet. The TEC should be set and active to stabilize the diode at a consistent, pre-determined, temperature (such as 25 °C).
3. Align the light output from the laser diode with the isolator.
4. Mount all steering mirrors with posts on the optical table. Additionally, it is suggested to mount the fiber coupling lenses on translational stages. Use the steering mirrors and the translational aspheric lens to couple the laser light into the single mode fiber (*see* **Note 3** for details on maximizing power into the AOM).

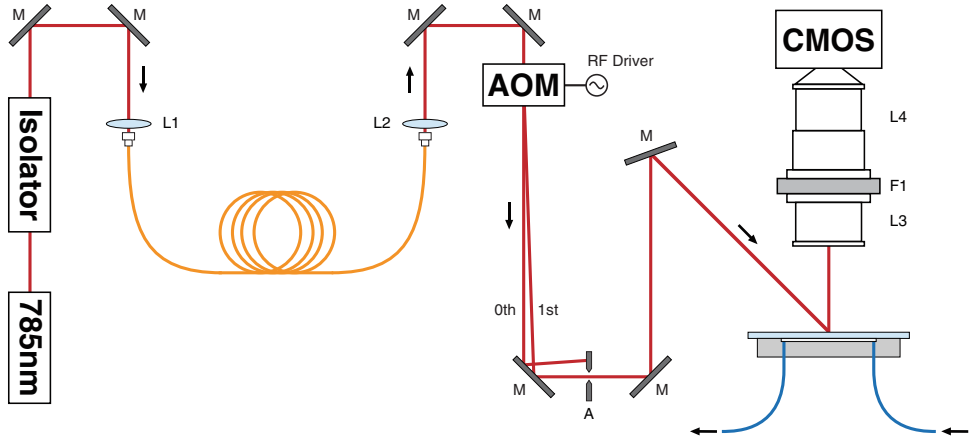

Fig. 1 A schematic detailing the connections for the optical components. The 785 nm laser diode is connected to the isolator, and mirrors (marked M in the figure) and a set of aspheric lenses (L1 and L2) are used to couple the light into a fiber-optic patch cable to obtain a Gaussian beam and to re-collimate the output from the single mode fiber. After passing through the AOM, the first-order diffraction is isolated using an iris (A) and is then relayed to obliquely illuminate the sample under test. Two lenses, L3 and L4, are used to collect the light, with a bandpass filter, F1, placed along the collection optics path. This filtered collected light is imaged onto the CMOS camera and contains the raw pixel intensity image data

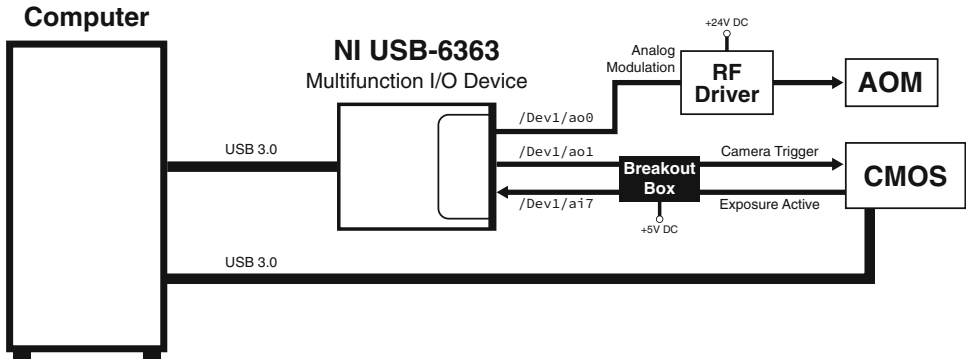

Fig. 2 A schematic of the connections required for the electronics**.** The DAQ serves as a communication hub between the workstation (lab desktop or clinical laptop) and the RF driver and the camera, generating the necessary voltages and trigger signals to modulate the laser light and the camera exposure times. The CMOS camera is connected to the workstation and transfers the image data back to the image collection software. A breakout box is used to supply a reference voltage to the DAQ

5. Use the second set of steering mirrors to direct the re-collimated light output from the end of the fiber into the AOM.
6. Use a third set of steering mirrors to direct light into the iris and isolate the first-order diffraction from the AOM.
7. Relay this first-order diffraction light to obliquely illuminate the sample under test.

3.1.2 Electrical Components

1. Use Fig. 2 to provide an overview of the connections between the various electrical components.
2. Connect the DC power supply to the RF driver. The model suggested in this chapter requires 24 V DC. If using a different AOM/RF driver, please see the manufacturer data sheet.
3. Connect the DAQ to the workstation via USB 3.0.
4. Connect the first analog output of the DAQ (/Dev1/ao0) to the RF driver of the AOM.
5. Connect the second analog output of the DAQ (/Dev1/ao1) to the camera trigger input using a 6-pin I/O plug.
6. Connect the camera to the workstation using a high-speed USB cable (such as USB 3.0).

3.2 Laser Alignment

Prior to any image acquisition, it is suggested to verify that the field of view (FOV) is properly illuminated. This section gives instructions on how to properly illuminate the imaging FOV.

1. Place the test object under the microscope (printed text is a good test sample).

2. Use the NI Device Monitor (in system tray of the connected workstation) to launch Test Panels for the DAQ.
3. Select the Analog Output tab, set the Channel Name to Dev1/ao0 (the channel that is connected to the RF driver), adjust the Output Value to ~0.05 V, and click Update to apply the change. This voltage can be adjusted as necessary between 0 and 5 V to increase or decrease the modulation of the laser light with the AOM.
4. Launch the image collection software to start a live view of the camera. Ensure that the laser light power into the AOM has been previously optimized (*see* **Note 3**) and that the illumination optics are correctly functioning.
5. Adjust the height of the microscope until the object is in focus.
6. Center the laser beam in the camera field of view by adjusting the knobs on the final steering mirror (the mirror that is used to illuminate the sample).
7. Close the image collection software and Test Panels programs.

3.3 MESI Calibration

The objective of the MESI calibration stage is to ensure that there is an equal amount of light at each exposure time. This is accomplished by controlling the amplitude of the analog voltage signal applied to the RF driver of the AOM and modulating this voltage based on the pixel intensity at each exposure time. There are numerous methods to solve this iterative process. The method implemented by our lab (*see* **Note 4** for complete details of the process) is described below.

1. Supply an initial guess voltage to the AOM. This guess should fall within the range of values that the DAQ can supply.
2. Record the average light intensity (pixel values) from this initial guess at each MESI exposure time (N total values, where N is the number of exposure times).
3. Iteratively, adjust the voltages at each exposure time to bring the intensities in line with each other. It is helpful to define an acceptable error tolerance. Additionally, it is useful to limit the maximum number of saturated pixels.
4. Save the voltages for each exposure time. These calibration voltages will be applied for any measurements that will occur with this sample.
5. Close the software.

3.4 MESI Acquisition

1. Open the image collection software to begin the MESI acquisition.
2. Set the number of MESI sequences to acquire and the desired output filename.

3. Apply the calibration voltages to each exposure time.
4. Record a sequence of MESI images. The image collection software should automatically cycle through the total number of sequences defined and save the speckle images.
5. Close the running software.

3.5 Shutdown

1. Set the laser current to 0 mA, deactivate the laser, and turn off the laser diode controller.
2. Deactivate and turn off the temperature controller.
3. Turn off the AOM power supply and reference DC voltage to the DAQ.
4. Remove the USB and I/O plug from the camera (prevents possible burn out).

3.6 Data Processing

Once the raw intensity images are obtained, it is necessary to extract the flow dynamics from the images. This section covers the primary steps in obtaining the ICT maps that are indicative of flow; additionally Fig. 3 provides a visualization of each step.

3.6.1 Calculating Speckle Contrast

1. Obtain speckle contrast: Convert the collected raw speckle data into the speckle contrast image (using Eq. 1). This can be done by iteratively solving Eq. 1 at every pixel in the collected images.
2. Set the speckle contrast window size, typically $N = 7$ (*see* **Note 6**).
3. Solve Eq. 1 and generate the speckle contrast images.

3.6.2 Imaging and Extracting Quantitative Flow Information

1. Use the computed speckle contrast, at each exposure time, to fit the measured data to the MESI equation (Eq. 3), and solve for the four unknown fitting parameters, including τ_c for a specific region of interest (*see* **Note 7**).
2. If a pixel-by-pixel map of τ_c is desired, then the fitting should be repeated at all pixels across the entire field of view from the measured speckle contrast vs exposure time.
3. Create ICT images to provide quantitative flow images that define the flow dynamics of the imaging scenario.

4 Notes

1. The LD785-SEV300 laser diode has a non-Gaussian beam profile. Optical correction of the abnormal beam shape is applied by coupling the light into an optical fiber. The laser was coupled into a single mode patch cable (125 μm cladding, P3-780A-FC-2, Thorlabs, Inc.) to obtain a Gaussian beam.

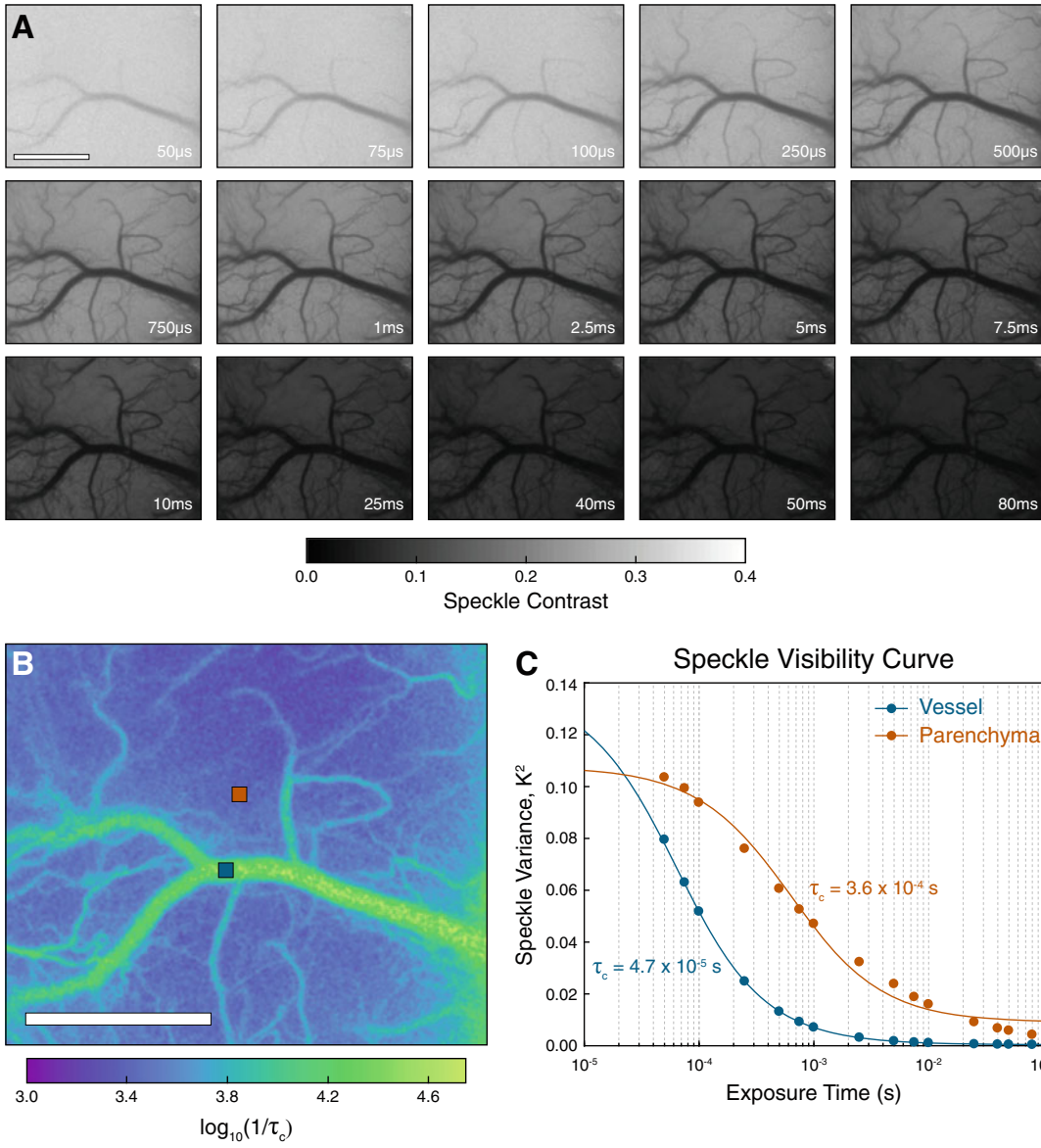

Fig. 3 (**a**) Example of the speckle contrast images of a mouse cortex (a cranial window is necessary so that the laser light reaches the brain surface) at all 15 MESI exposure times, (**b**) the resulting ICT image (log scale) for this set of MESI images after solving for τ_c at each pixel of the image, (**c**) plots of the MESI fits in each ROI from (**b**) with the calculated τ_c in each region. Note that the ICT values are much higher in the vessel, where blood flow is significantly higher, as opposed to the parenchyma, which is a region of comparatively low blood flow. (Image reproduced from [19])

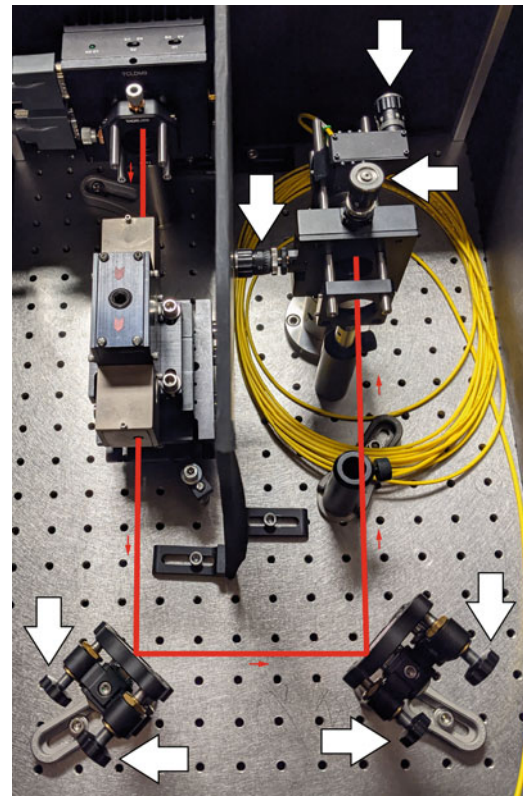

Fig. 4 Example of the light path to optimize to maximize power delivered to the system. The smaller red arrows indicate the path the illumination light follows, whereas the white arrows indicate the steering mirrors and the translational stages that can be used as control parameters to optimize the light delivered to the sample under test

The fiber output was re-collimated (F230APC-780, Thorlabs, Inc.) before being relayed to the free space AOM.

2. Maximize the power output of the system by ensuring that light is properly coupled into the fiber-optic cable. The following steps and Fig. 4 help this process:
   (a) Place a post-mounted optical power sensor in the holder downstream of the collimated fiber output. Center the laser beam on the sensor surface. *Only use power sensors designed for your laser diode choice*. Set the power meter to a wavelength of 785 nm (or if not using a 785 nm, match the wavelength of your optical source).
   (b) Using the power meter as reference, adjust the steering mirrors and translation stage for the fiber coupling lens to maximize the fiber output power. If necessary, the translation stage for the laser collimating optic can be adjusted as well. ***Warning:*** *Be careful working around the free-space*

laser beam. Consider reducing the current on the laser diode controller during this step.

(c) Once the fiber output power has been maximized, remove the optical power sensor.

(d) See Fig. 4 for an example of a system layout and the various steering mirrors and the translational stage that can be modified to optimize power.

3. It is highly suggested to create an image collection software that will allow for interaction with the suggested Basler cameras, the workstation, and the DAQ to acquire and save the MESI images. The pylon Camera Software Suite contains easy to use APIs, a viewer for live camera evaluation, and the drivers to interact with the Basler cameras. It is possible to develop dedicated software that can allow new users to easily acquire MESI images. Some suggestions for the development for this software are as follows.

4. Our lab has implemented a MATLAB (MathWorks, Inc.) script that uses the ANSI C NI-DAQmx library (National Instruments Corp.) to operate the DAQ to produce both the camera exposure trigger signals and AOM modulation voltages necessary for the MESI calibration stage (Fig. 5). While this functionality can be implemented directly in the acquisition software itself without relying on MATLAB, the concepts and methods for implementing the calibration defined here can be used. The camera exposure trigger signal and the AOM modulation voltage waveforms are both generated at 1 MHz with identical pulse durations that match the chosen camera exposure times. A slight temporal offset, +25 μs delay for the AOM

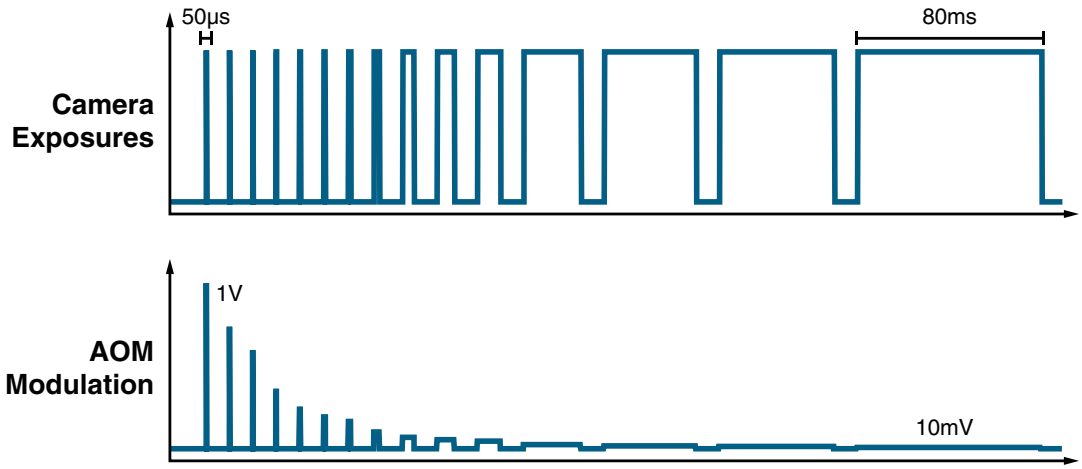

Fig. 5 An example of the timing diagram for the MESI control signals. In this example we can see the 15 pulsed camera exposure trigger signals that make up a MESI frame and the corresponding modulation voltages that are applied to the RF driver for each specific exposure time. (Image reproduced from [19])

signal, is used to guarantee that the actual camera exposures and the laser pulses were synchronized in time. The AOM modulation voltages for each exposure are determined using the calibration procedure outlined below:

(a) An initial guess, between 0 and 1 V, for the modulation voltages is generated using a power law function.

(b) These voltages are used to acquire a complete MESI frame containing 15 raw intensity images from different exposures.

(c) The average intensity and total number of saturated pixels within a user-defined region is then calculated for each image. If the overall coefficient of variation and the number of saturated pixels is less than the defined thresholds, then the intensities are equalized and the calibration is complete. The thresholds define the tolerance of dissimilarity between the average pixel intensity at each exposure time.

(d) If the average intensity variation is too high or if there are too many saturated pixels, then each of the modulation voltages are adjusted accordingly using the shortest exposure time as the target intensity.

(e) This process repeats recursively until the stop conditions are achieved.

(f) A typical calibration will take between 30 and 50 iterations and complete within less than a minute.

5. The exact choice, and number of exposure times, remains a topic of research. While the majority of the MESI studies from our lab have focused on using 15 exposure times spanning three decades of exposure times from 50 μs to 80 ms, the crucial aspect is to fully capture the MESI curve. The longer exposure times help us resolve the MESI curve in scenarios with low flow and slow changing dynamics, and the shortest exposure times are needed for higher flow and quickly changing dynamics (the lowest exposure time will be hardware limited by the camera and AOM minimum gating times). The range and number of exposure times can be further optimized for specific applications; it is possible to optimize the exposure times if a priori information about the sample or test case is known. This can help speed up MESI acquisition and move it closer to being a live, real-time imaging tool.

6. Computing speckle contrast: To compute the full speckle contrast image, as defined by Eq. 1, we must first define the NxN sliding window. Statistical analysis [1] and past precedent [18] have established $N = 7$ as an optimal window size to ensure both image resolution and speckle contrast sampling. A

properly sampled speckle pattern will produce speckle contrast values ranging between 0 and 1 [6]. Iteratively go through each of the collected raw images and compute the speckle contrast images for each exposure time. These speckle contrast images are required to solve the ICT maps.

7. Levenberg-Marquardt nonlinear least squares optimization is used to fit the unknown MESI parameters (ρ, τ_c, ν, β) to the computed speckle contrast data collected from the experiment. This runs on a pixel-by-pixel iterative basis. Loop through the entire image to get the complete τ_c map (*see* **Note 8** about initial guesses and the choice of β).
8. The β parameter is an instrumentation factor that theoretically should remain constant so long as the illuminating light and imaging optics are unchanged. However, if β is fitted for at every time point, it could vary significantly over time, impacting the stability of ICT. Microfluidics tests have shown that holding β fixed results in a more robust reproduction of stepped flow profiles. Therefore, when processing time-resolved MESI data, it is advantageous to perform a preliminary fit to estimate the value of β. Solve for beta over pre-defined region (e.g., the first 100 frames), and use this for subsequent analysis. Additionally, an initial guess for ρ, τ_c, ν is typically included in the nonlinear least squares optimization, and the initial guess can impact the speed at which the fit reaches a solution. An alternative to this fitting method is something like using a genetic algorithm to solve for the MESI parameters.

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