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Aligning the μs-ALEX Setup

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To achieve single-molecule sensitivity and thus have the ability to detect single diffusing fluorophores, careful alignment of the microsecond-alternating laser excitation (μs-Alex) setup is crucial. The following protocol describes routine alignment for 2c-ALEX (532 nm/635 nm) with spectral windows $G^{550-620}R^{650-750}$.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Alignment sample (e.g., tetramethylrhodamine [TMR; Invitrogen] or Cy3B [Amersham Biosciences])

Equipment

Avalanche photodiodes (APDs)
Camera and closed circuit TV (CCTV) monitor (optional; see Step 3)
Coverslip
Lasers
> *Extreme caution is required when using lasers.*

Microscope with appropriate components
Optical density (OD) filter (flippable) (optional; see Step 3)

METHOD

1. Prepare the alignment sample (concentration of $\sim 10^{-9}$ M) of a fluorophore that can be excited with 532 nm and shows considerable emission cross talk on the red channel (and thus allows alignment of both detectors).
    > *Solutions of TMR or Cy3B are well suited.*

2. Set laser power of the green laser to ~ 50 μW (measured right before entering the microscope).
    > *This should yield a considerable signal in the range of 50–100 kHz. If the signal is lower (e.g., owing to setup misalignment), increase the laser power until you reach the desired count rate.*

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3. Deliver 50 µL of the alignment sample onto a cover slide. Using the eyepiece, find the focus of the glass–water interface, and raise the objective to move the focused beam ~20 µm into the solution.

> *Laser radiation is dangerous for the eyes. Whenever eyepieces are used and laser intensities are >100 µW, the beam should be attenuated before entering the microscope (e.g., with a flippable OD filter). Alternatively, a simple camera mounted on the camera port and connected to a CCTV monitor can be used for focusing.*

4. For the first alignment, remove the pinhole and align the detectors using the micrometer screws of an *x–y* stage.

> *If the lenses in front of the APDs are positioned accurately and the beam is collimated, you should observe a plateau for the detection response of the APDs (owing to the small size of the focus compared with the active area of the APD).*

5. Insert the pinhole and align its position using the *x–y* positioning stage. At the first alignment, ideally start with a larger pinhole (200 µm), followed by 100 and 50 µm.

> *It is important to ensure that correlated signals on both detectors are observed when the pinhole is repositioned to identify its best position.*

6. Align detectors again using the procedure outlined in Step 4.

> *It is convenient to establish a range of expected observables after the alignment procedure for a particular setup; for example, this can be the count rate for a standard concentration of an alignment sample (10^{-9} M of a desired fluorophore) and a defined excitation power (50 µW). The important values are the detection ratio between the two detection channels, as well as the count rate of emission.*

RELATED INFORMATION

See **Alternating Laser Excitation for Solution-Based Single-Molecule FRET** (Kapanidis et al. 2015a), **Assembling the µs-ALEX Setup** (Kapanidis et al. 2015b), and **Sample Preparation and Data Acquisition for µs-ALEX** (Kapanidis et al. 2015c).

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