Title
Sample Preparation and Data Acquisition for µs-ALEX.

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Protocol

Sample Preparation and Data Acquisition for µs-ALEX

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This protocol describes the preparation of samples and data acquisition for microsecond-alternating laser excitation. Sample preparation requires a dilution that ensures the detection of single events.

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

Bovine serum albumin (BSA)
Buffer components appropriate for sample (specified "for luminescence spectroscopy," e.g., from Fluka, Merck, or Fisher Scientific)
KOH (1 M)
Sample to be assayed

Equipment

Coverslips
Filters (0.2-µm)
Gaskets (sealable plastic) or enclosable chambers
Lasers

Extreme caution is required when using lasers.

Microscope with appropriate components for data acquisition

METHOD

1. Clean coverslips with 1 M KOH and rinse with H_2O before use.
2. Prepare buffer and filter it through a 0.2-µm sterile filter. Measure the buffer alone with settings identical to those used for sample data acquisition, to decide on purity.

   See Troubleshooting.

Adapted from *Imaging: A Laboratory Manual* (ed. Yuste). CSHL Press, Cold Spring Harbor, NY, USA, 2011.

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3. To avoid adsorption of biomolecules to the coverslip (e.g., large proteins that tend to stick to glass), BSA (10–50 µg/mL) may be added to the buffer. Either pretreat surfaces with BSA-containing buffer (the protein will form a layer on the glass surface), or perform measurements using BSA-containing buffer.
4. Dilute the sample in buffer to a concentration of 100 pM.

   Typically, the confocal volume is ~1 fL, and a concentration of 100 pM results in a probability of 0.1 to find a single molecule within the confocal volume.
5. Use sealable plastic gaskets or enclosable chambers to perform measurements.

   This helps avoid solvent evaporation during data acquisition, which could cause a subsequent increase of sample concentration.
6. Acquire data using appropriate settings and analyze.

   See Troubleshooting.

TROUBLESHOOTING

Problem (Step 2): There are impurities in the buffer.

Solution: Buffers for single-molecule experiments need to be free of fluorescence impurities. The number of fluorescence impurities depends strongly on the quality of the chemicals used for buffers. Many of those chemicals are available in high purity and should be used for the preparation of measurement buffers. Buffer purity is best evaluated by the number of bursts observed, which should be considerably lower than the sample itself, and of lower intensity. In general, impurities will appear on the green channel (as impurities showing fluorescence in the red spectral region are rare), and thus can be filtered out during data analysis. In the actual experiment, the fluorescence count rate of impurities should be considerably lower (use less than about one-third as a rough guide) than for the fluorophores used, and the burst frequency (which is proportional to the concentration of impurities) should be <10% compared with a 100 pM solution of labeled molecules.

Problem (Step 6): Photon yields are inadequate.

Solution: The total photon yield of organic fluorophores is limited (~10^6 photons emitted before photobleaching). Depending on the chemical class of fluorophores, oxygen removal (Yildiz et al. 2003) and addition of triplet quenchers to the buffer solution (Rasnik et al. 2006) can increase photon yields significantly.

Problem (Step 6): Photobleaching occurs.

Solution: High excitation powers will lead to photobleaching of the fluorophores (both donor and acceptor). In fluorescence resonance energy transfer (FRET) experiments using an acceptor excited by 635-nm light, acceptors are more sensitive to photobleaching. In some cases, photobleaching can be reduced by modifying the usual 50%–50% green–red excitation duty cycle into an excitation cycle that allocates 80% to green excitation and 20% to red excitation (Kong et al. 2007). Photobleaching can equally be dealt with using ROXS (reducing and oxidizing system) buffers, at least to a certain extent.

Problem (Step 6): There are insufficient statistics to answer the question at hand.

Solution: µs-ALEX in solution detects single events and derives statistical data out of them. Any experiment should aim at sufficient statistics to answer the questions. Use measurement times from 2 to 30 min. Fast measurements provide a quick view of the sample and allow decisions on whether a longer trace is needed. Proper sealing of the sample, reliable temperature control, use of suitable excitation power (e.g., to allow sufficient photon counts while preventing significant photobleaching), and a physically and optically stable setup are important for ensuring constant conditions for long acquisition times.

Problem (Step 6): Inaccurate results are obtained.

Solution: Record additional experimental variables for proper data analysis. Determine the background on each detector (green, red) during the two excitation cycles (red or green excitation) from buffer-only measurements. At the same time, determine the donor leakage (relative emission of the donor fluorophore on the acceptor channel) and direct excitation of the acceptor by using single-labeled constructs under identical conditions.

Problem (Step 6): Incorrect *E* values (FRET efficiency) and distances are obtained.

Solution: If experiments aim to determine exact *E* values (and corresponding distances), determine the correction factor γ for the set of fluorophores used (see **Alternating Laser Excitation for Solution-Based Single-Molecule FRET** [Kapanidis et al. 2015a]).

Problem (Step 6): There is inadequate resolution of subpopulations.

Solution: The ratio of the excitation powers will influence the stoichiometry ratio *S* (see **Alternating Laser Excitation for Solution-Based Single-Molecule FRET** [Kapanidis et al. 2015a]). Changing this ratio will influence the separation of populations in the 2D *E*–*S* histogram and thereby affect the resolution of subpopulations.

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 **Aligning the µs-ALEX Setup** (Kapanidis et al. 2015c).

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