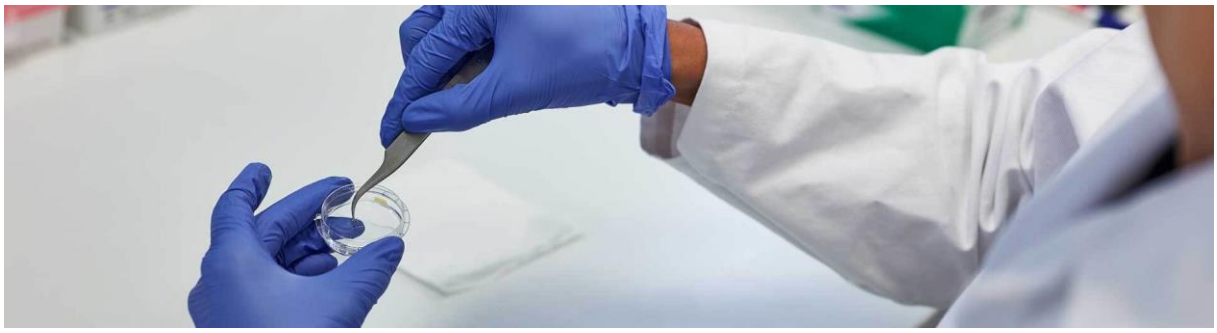
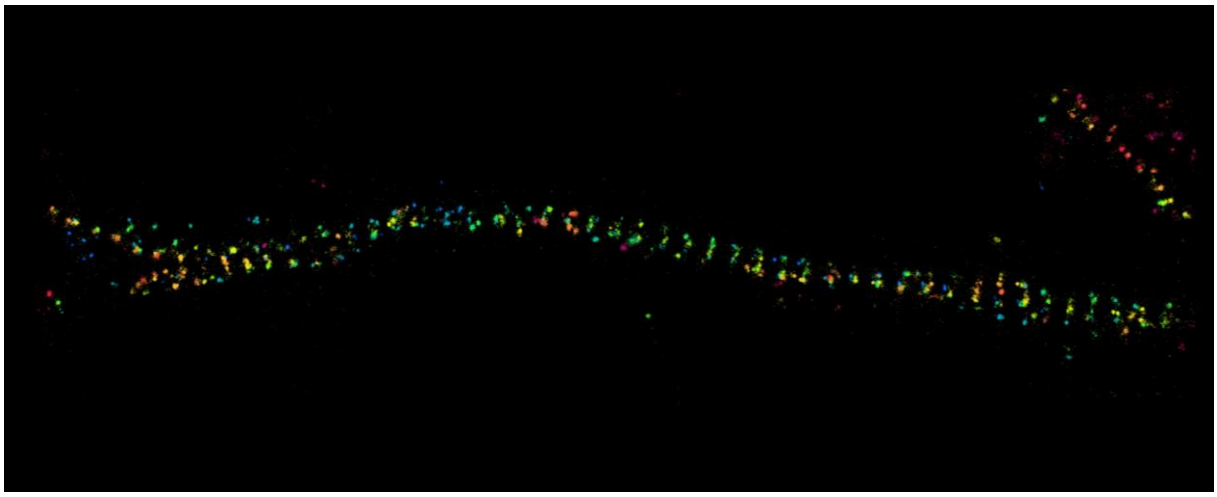
MINFLUX Microscopy Sample Preparation





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While much of the sample preparation process can proceed as for STED, certain MINFLUX-specific factors must be taken into account for best results. As a young technique, new labels and sample preparation methods are regularly being developed and reported. Here we summarize our current MINFLUX sample preparation recommendations.

1. Before You Label

#1.5 or **#1.5H glass coverslips** should be used in all cases. **Do not** use plastic or #1 coverslips. If possible, please **avoid gridded coverslips**, as the grooves may affect the focus.

As for STED or any other advanced microscopy technique, care should be taken to optimize cell growth and fixation to minimize artefacts and improve overall sample quality.

Background can be a particular issue in MINFLUX acquisitions, so a quenching step to reduce autofluorescence – using, for example, **ammonium chloride, glycine, or sodium borohydride** - may also be incorporated where aldehyde fixation protocols are used.

For complex samples, our MINFLUX Application team (application@abberior-instruments.com) will be happy to discuss your sample preparation.

2. Choosing Labelling Strategies for MINFLUX Imaging

2.1. Single Molecule Strategies

For MINFLUX, only a **single emitting fluorophore** is allowed to be inside the probing pattern at any one time, so any labelling strategies employed must allow fluorescence at a single molecule level. There are several options that have been demonstrated since MINFLUX's inception. This section will cover the main strategies currently available and give some specific label recommendations.

2.1.1. Carbocyanines

One of the most popular strategies for single molecule localization microscopy is to use carbocyanines, like Alexa Fluor 647. When adding a reducing agent - for example a thiol component like cysteamine (MEA) - to an imaging buffer, the reducing agent binds to the fluorophore in its excited state and renders it in a non-fluorescent dark state. An appropriate oxygen scavenging system is also required to limit the quenching of triplet states and photobleaching. After initial excitation, the majority of molecules are pushed into this dark state, with single fluorophores spontaneously switching back to fluorescent form. This back reaction can be prompted by activation with 405, resulting in tunable stochastic blinking at the single molecule level.

Recommended carbocyanine dyes:

640 nm - *abberior* FLUX dyes (FLUX 640, FLUX 647, FLUX 660, FLUX 680), Alexa Fluor 647, sCy5, CF660C, CF680.

2.1.2. Photoswitchable or Photoactivatable Dyes or Proteins

Broadly, these fluorophores are in a resting state – either non fluorescent (photoactivatable) or fluorescing at a lower wavelength (photoconvertible) until activation using UV light to a MINFLUX appropriate fluorescent form. As this activation or conversion is controllable, the 405 power applied can be tuned to activate at a single molecule level. The photoconvertible fluorescent protein mMaple is one example that has been demonstrated in MINFLUX.

2.1.3. DNA-PAINT

DNA-PAINT uses binding kinetics of short DNA oligomers rather than manipulating photophysics to generate the 'blinks' at the labelled epitope. The protein of interest is labelled with a docking strand, with the complementary 'imager' oligomer labelled with a fluorophore binding and unbinding over short timescales. The imager fluorophore should be bright and photostable – avoid blinking dyes and conditions under which dyes can show a blinking behaviour.

Recommended imager dyes:

640 nm - ATTO 655

561 nm – Cy3B

2.1.4. Spontaneously Blinking Dyes

Dyes such as HM-SiR, a spontaneously blinking silicon-rhodamine dye, can also be used in MINFLUX. Self-blinking labels specifically designed for MINFLUX imaging have recently been reported. This labelling approach can have benefits, as blinking is not dependent on activation or a reducing blinking buffer.

2.1.5. Suitable Costains

In addition to a MINFLUX appropriate label, you can also include **spectrally non-overlapping labels** as a confocal reference (e.g. GFP, YFP, *abberior* STAR GREEN, Alexa Fluor 488, etc.).

☞ If using a carbocyanine or UV dependent photoswitchable/convertible MINFLUX label, **do not** costain with DAPI or other dyes excitable at 405 nm.

2.2. Multicolor Experiments

Two color labelling can be performed with carbocyanines, using one excitation line and two dyes that are spectrally distinguishable. Detections are split over two APDs relative to emission wavelength, and therefore the ratio of photons detected on each APD can be used to generate a two color image. This ratiometric approach to multicolor imaging allows one-step acquisition of two color data. Ideal fluorophore pairs will blink well at similar thiol concentrations. Before designing a two color experiment, please test and optimize each label in your sample in single color acquisitions. It is important that both dyes blink well in the same buffer – some spectrally feasible fluorophore combinations require very different concentrations of reducing agent for optimal performance, limiting application. The below pairs have been shown to perform well when combined.

Recommended fluorophore pairs:

abberior FLUX 640 & FLUX 680

CF680 & sCy5

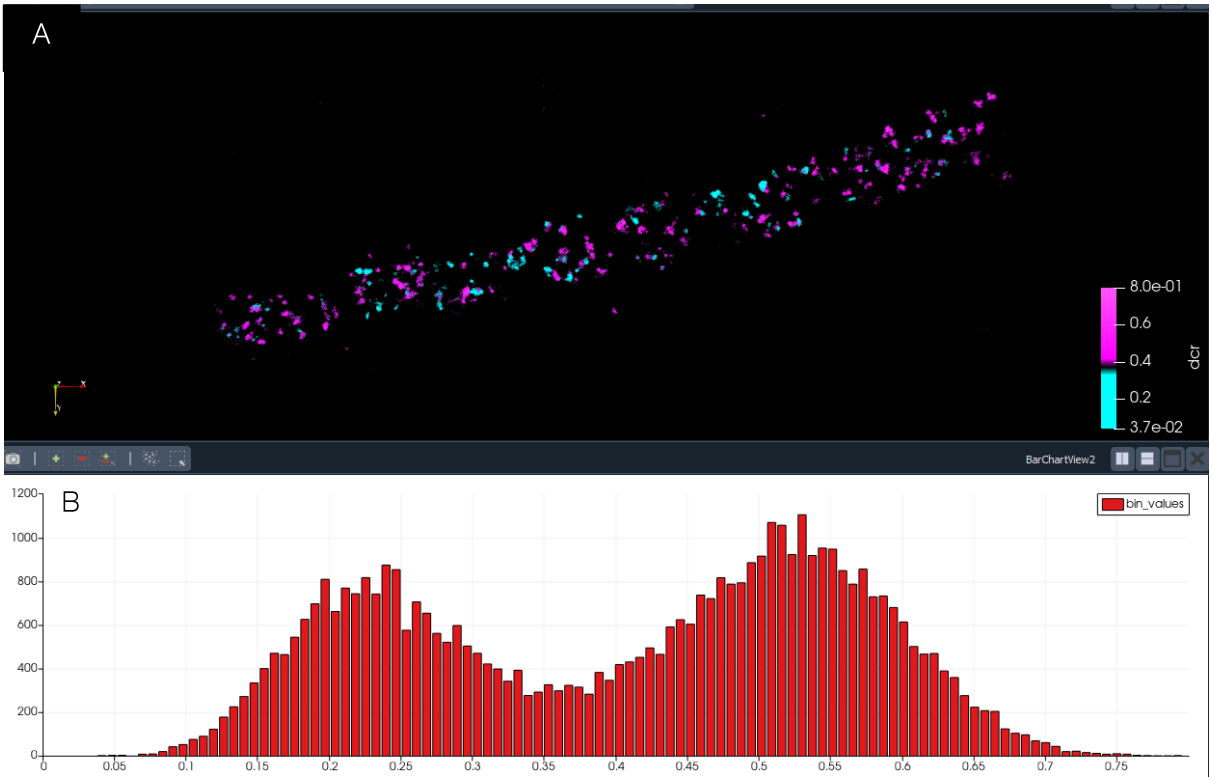

Figure 1 – Example two color data, showing TOM20 labelled with sCy5 (magenta) and mitofilin labelled with CF680 (cyan) in panel A. Panel B shows the detection channel ratio values from this dataset, showing the clear spectral distinction.

Multicolor imaging is also possible using exchange-PAINT. Samples are labelled with different docking/imager strand pairs for each epitope. Imager strands are exchanged and images are acquired sequentially whilst maintaining the same reference position in the stabilization system.

2.3. Label Properties and Label Size

While MINFLUX can be performed successfully with standard indirect immunofluorescence labelling, it is important to **consider the size of your label**, especially relative to the size of your structure of interest. Typical primary/secondary antibody labelling can result in a significant distance between the epitope and the fluorophore, termed 'linkage error'. This distance is often greater than the resolving power of a MINFLUX system, and could obscure smaller structures. (See also: Früh et al., ACS Nano, 2021)

In addition, fluorophore stoichiometry can also be a key consideration in some applications – standard secondaries can have varying numbers of conjugated fluorophores, as well as potentially having multiple secondaries binding to each primary, meaning the same epitope may be repeatedly relocalized, or the label density may be too high to permit single molecule imaging.

It is worth considering using **small self-labelling enzymes** like SNAP or HALO tags, nanobodies, or other target specific small probes.

MINFLUX, like all single molecule methods, is also very sensitive to non-specific label – you may find that you need to increase your blocking step incubation times, increase the number and length of wash steps, or reduce your antibody concentration.

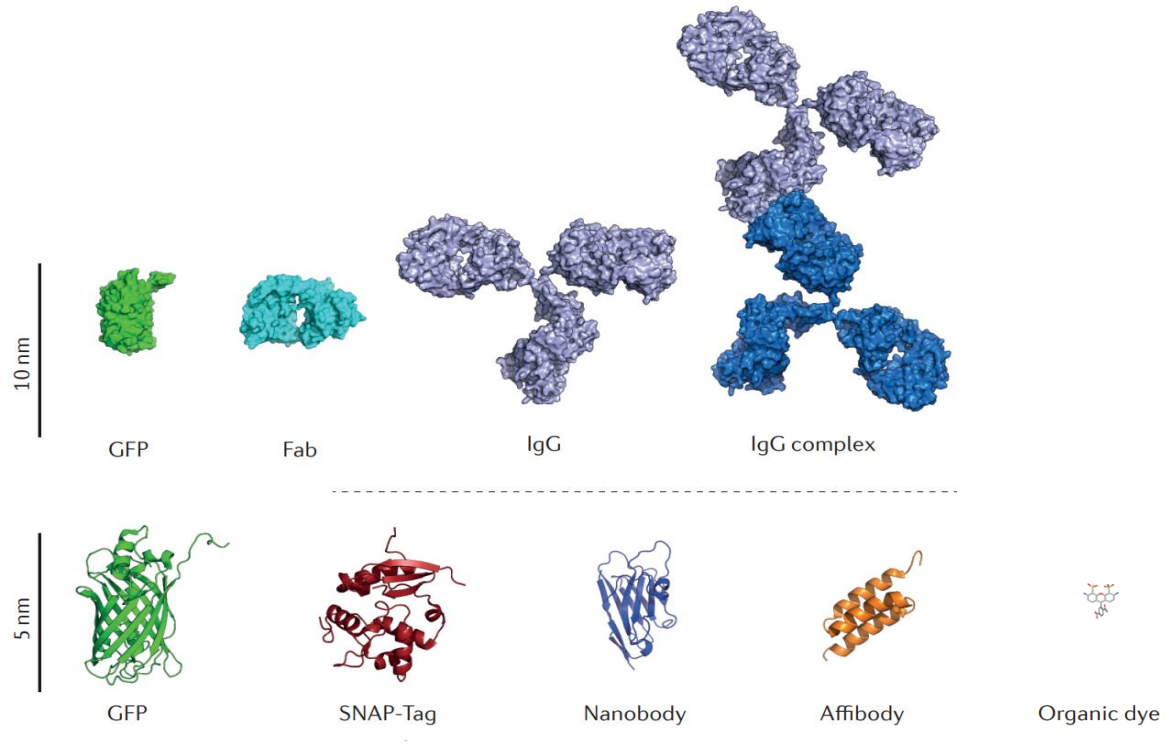

Figure 2 – Illustration of common label sizes. Adapted from Sahl et al., Nat Rev Mol Cell Biol., 2017.

3. Sample Preparation for MINFLUX Imaging

3.1. Stabilization

Imaging samples must be incubated with gold fiducials – beads or nanorods – for the MINFLUX stabilization system. These fiducials can be introduced either during initial coverslip preparation, if tolerated by your sample, or by incubation with the fixed and labelled sample. For a standard 18 mm coverslip, the sample should be incubated with 100 µl of undiluted fiducial solution for 5 minutes. After discarding the solution, 3 brief PBS washes followed by a 10 minute wash step with PBS containing 10 mM $MgCl_2$ can help to remove shaking or rolling beads prior to mounting. Additional care should be taken at this step with any samples that can become easily dislodged, such as cultured neurons or non-adherent cell types.

☞ Make sure to **thoroughly mix** your fiducials before use!

3.2. Blinking Buffers

As described above, when using a carbocyanine to label your sample, blinking is made possible using a buffer containing a reducing agent and an oxygen scavenging system. A commonly used reducing agent is MEA. Oxygen scavenging systems are often composed of an enzymatic system of

glucose oxidase and a catalase (commonly referred to as GLOX, or a GLOX buffer), though other systems have been reported.

Preparation of GLOX buffer

Blinking buffer base: 50 - 100 mM Tris-HCl, 10 mM NaCl, 10% (w/v) Glucose, pH 8.0

Component	Stock concentration	Final concentration (in buffer base)
Catalase	20 mg/mL in dH2O	64 µg/mL
Glucose oxidase	70 mg/mL in 50 mM Tris-HCl (without glucose)	0.4 mg/mL
MEA	1 M in PBS	5 - 70 mM

The buffer base can be prepared in advanced and stored at room temperature. Enzyme stock solutions can be stored for several weeks at 4°C. Aliquots of MEA stock solutions should be frozen, with a new aliquot thawed every 3 – 4 daysBlinking buffers, especially those with enzymatic components, should be prepared fresh before imaging. In an environment sealed against oxygen, the buffer can function for between 4 and 8 hours. If the sample is unsealed, this working time will be greatly reduced.

☞ Optimize the concentration of the reducing agent for your particular fluorophore and the fluorophore density in your sample for best results.

For PAINT acquisitions, it is important to optimize your imager strand concentration. More imager strand will result in more frequent 'blinks', but can also result in too many fluorescing molecules in close proximity. High imager strand concentrations can also significantly increase background, as the fluorophore will still fluoresce when in the buffer unbound. Adjusting the imaging buffer composition and the docking/imager strand pairs can also affect the length of time the imager strand remains bound, and thereby regulate imaging speed (Schueder et al., Nat Methods, 2019).

☞ Aim for an appropriate blinking density and background for MINFLUX while not overly reducing imaging speed.

3.3. Mounting

Mounting will vary depending on your sample and application. At *abberior*, we usually mount samples with the appropriate blinking buffer on cavity slides, which are then sealed with a 2-part silicone glue (figure 3). This forms a seal to prevent oxygen access during imaging but is removable so that the sample can be remounted if necessary. Under these conditions, GLOX buffer tends to last around 6-8 hours.

If performing, for example, exchange-PAINT imaging, glass bottomed dishes or larger circular coverslips (e.g. 25 mm) in an imaging chamber can also be used. If using a multiwell slide, please do not use the outer wells, as the sample holder will not allow sufficient travel of the objective to reach them.

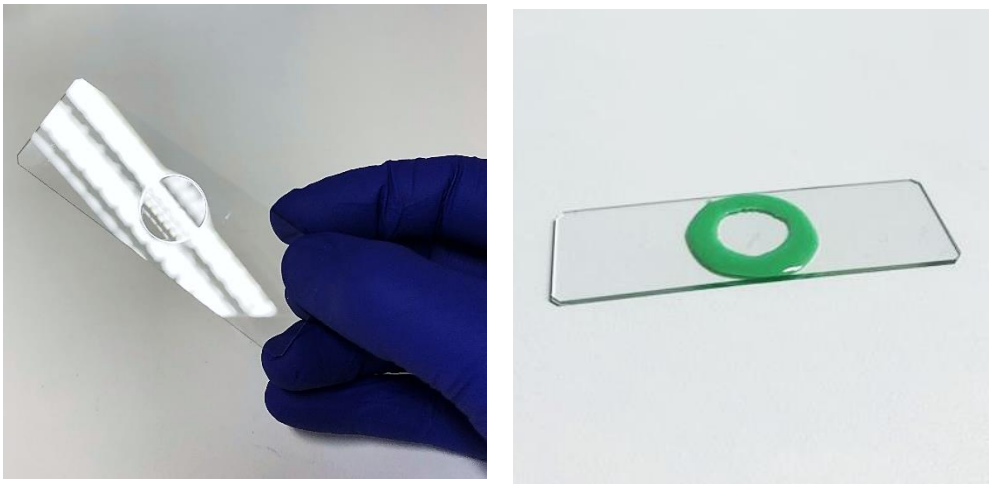

Figure 3 – Cavity slides used for mounting sample coverslips with two-part silicon glue.

3.4 Sample Storage

Prior to imaging, fixed samples can be stored in buffer at 4°C, as for standard immunofluorescence samples. Gold fiducials should be added to the sample before mounting. Previously mounted samples can be stored at 4°C for short periods (<5 days) before remounting and reimaging, but fiducial stability can reduce with time. For immunostained samples, especially with single domain antibodies, consider post-fixation to prolong sample stability.

4. Sample Preparation for MINFLUX Tracking

4.1. Labelling Strategies

Self labelling enzymes, photo-switching fluorescent proteins, small organic probes, and click chemistry are popular labelling approaches for tracking. Position of the epitope within the cell should be considered when selecting a tag – some self-labelling enzyme ligands are cell impermeable. Antibodies and nanobodies may be useable for some cell surface epitopes.

4.2. Labelling Density

Labelling density is critical for tracking experiments. Individual labels must be sufficiently well separated to allow accurate MINFLUX tracking. An appropriate labelling density could be reached with:

- Targets that are expressed in low copy numbers, either endogenously or artificially low expression.
- Dilution of fluorophore to label only a proportion of the existing target – often in the picomolar range.
- Use of photoconvertible/activatable fluorescent proteins or dyes.

In all cases, excessive over-expression should be avoided.

4.3. Fluorophores

While a blinking fluorophore is ideal for fixed imaging, fluorophores used for tracking experiments should be bright and photostable to permit tracking over time, such as:

640 nm - ATTO 647N, *abberior* STAR RED, JFX 650, JF646

561 nm – JFX 554, mMaple/mEOS3.2 (photoconvertible)

Beads or quantum dots that fluoresce in the appropriate wavelengths can also be trialled.

4.4. Sample Preparation

As for imaging, samples should only be prepared on **#1.5** or **#1.5H glass**.

Samples can be prepared on coverslips or in glass bottomed dishes. If using a multiwell slide, please do not use the outer wells, as the sample holder will not allow sufficient travel of the objective to reach them. Square or rectangular coverslips can also be used to build flow chambers using scotch tape.

4.5. Mounting

Live cells should be mounted in a phenol red free cell medium, like FluoroBrite or MEMO. In vitro tracking experiments can incorporate a triplet quencher (e.g. TEMPO) and/or ROXS agents (e.g. AA/MV, TX/TX-Q).