**STORM Considerations**

STORM is a Single Molecule Localization Microscopy (SMLM) method in which single molecules of fluorescence (isolated emitters) are imaged and the software is then used to locate the center (centroid) of the emitter’s Point Spread Function (PSF).

STORM requires a high-contrast, bright fluorochrome signal imaged against a dark background. For effective STORM, try to maximize the number of photons per emitter while minimizing background.

Remember, the resulting STORM data does not provide a true image but a “Pointillist” data set, representing a 2D/3D array of points corresponding to the dye molecule’s calculated centroid.

**Probe Properties**

1. **Photon Output** – brightness of the probe

Each captured photon produces an independent measure of the center of the probe position.

Detection of many photons during a single “blink” is required for precise measurement of the center of the probe position.

Precision of localization = optical resolution/square root of # photons emitted from single point

For 10nm accuracy, at least 1,000 photons per probe location is required (assuming negligible background). Precision of location does not equal optical resolution.

Fluorescent Proteins are not as bright as synthetic dyes, which can affect localization of FPs.

High precision of localization requires a large number of photons to be emitted in the ON-State.

1. **Duty Cycle**

Ratio of the time the probe spends in ON-State / OFF-State.

High duty cycle, meaning the probe stays ON for a long time, would increase the probability that two adjacent probes would be ON at the same time (probe overlap/blink overlap) and would decrease the accuracy of location.

Low duty cycle, meaning the probe stays ON for a very short time, would increase the probability of imaging one probe at a time. However, if the probe is only ON for a short time, a high number photons would need to be produced during the short ON time.

Duty Cycle is dependent on Labeling Density. Samples with high label density may need probes with low Duty Cycle to avoid probe overlap. Samples with sparse label density can use probes with higher Duty Cycle since there is low probability of overlap.

Duty Cycle may also be dependent on the imaging buffer, exposure time, laser power and laser illumination angle.

Best configuration would be a low Duty Cycle with a high # photons.

1. **Switching Cycles or Fatigue Rate**

Number of times a probe can cycle on/off before it is permanently photobleached.

Highly dependent on the chemistry of the buffer that the probe is in.

Synthetic (organic) dyes typically have a very high number of switching cycles.

Fluorescent proteins will typically have only one switching cycle per molecule (photoactivation ON / photobleach OFF).

Best probes would have a high Switching Cycle.

1. **Survival Fraction or High Photostability**

Number of probes capable of switching divided by the number of probes photobleached after imaging for 400 seconds.

Best probes would have a high Survival Fraction.

1. **Contrast Ratio** – residual dark-state fluorescence

Intensity of the probe after photoactivation (ON) divided by the intensity of the probe before photoactivation (OFF). The background of the image can increase due to residual fluorescence of the probe in the dark state.

Best probe would have a high Contrast Ratio.

1. **Laser Power**

OFF rate is proportional to the Laser Power.

Excitation laser power should be set as high as possible (100%). For low mW lasers, available laser power may not be sufficient to send dye to the OFF State but instead will increase dye photobleaching. For STORM, lasers should typically have >200mW at the fiber.

Fluorescence Proteins photoactivate at lower laser power.

1. **Labeling Density or Sampling Rate**

The antibody concentration should be at a level that just saturates the available binding sites. Saturation of the sample with primary and/or secondary antibodies can lead to non-specific binding. Unbound or excess dye should be avoided since this will cause high background.

If structure of interest is very dense, then incubation times/concentrations should be reduced to prevent detection of overlapping probes (probe overlap).

Fluorescent Proteins by design have a 1:1 label and no non-specific labeling.

Ideal Labeling Density is 1:1.

Fluorescent labeling density should be at least twice the intended resolution (Nyquist). To achieve 40nm lateral resolution, you would need 20nm localization precision. Resolution will depend on many factors including the density (number/proximity) of the fluorescent probe, the size of the attached probe (antibody versus fluorescence protein), antibody concentration, sample fixation, probe performance (# photons, duty cycle, buffer, etc.), and other factors.

1. **Label Size**

A small probe size will allow probes to be spaced closer together which, in theory, would permit better resolution. However, if the probes are spaced very close together, then a very low Duty Cycle would be required to prevent probe overlap.

Primary + Secondary Antibody = 20nm

Direct-Labeled Primary Antibody = 10nm

Fab Fragment = 3nm

Fluorescent Protein = 3nm

SNAP/Halo Tags = 3nm

Nanobody (single domain antibody) = 2nm

Aptamer (oligonucleotide or peptide) = 2nm

Synthetic Dye – 1nm

**Probe Classifications**

**In general for STORM, red-shifted dyes perform better than blue-shifted dyes.**

1. **Activator-Reporter**

Activator – non-radiative energy transfer by the activator probe to the reporter probe facilitates the return of the reporter from the OFF-State (T1) to the ON-State (S0).

Reporter – fluorescent dye that will be imaged

Examples:

Cy3-Cy5 – pulse activate at 560nm while continuously imaging at 647nm

Alexa488-Cy5 – pulse activate at 488nm while continuously imaging at 647nm

1. **Advantages of an activator-reporter dye pair**

For multi-probe recordings, by using the same fluorochrome for both reporters (e.g., Cy5), there are no concerns about buffer compromises or chromatic aberrations.

1. **Limitations of an activator-reporter dye pair**

For multi-probe recordings, since most reporter fluorochromes can also be activated in the absence of the activator (dSTORM), if the same reporter is used for both probes (e.g., Cy5), it can be difficult to determine the difference between each Cy5 emission for the two different probes and can lead to high levels of inter-channel cross-talk.

In addition, Activator-Reporter dye pairs are not commercially available, so they must be generated in-house.

1. **Disadvantages of dSTORM (direct activation of the reporter)**

For multi-probe recordings, there may be a need to compromise the optimal buffer conditions due to the use of two different fluorochromes, resulting in poor STORM performance for one or both dyes.

Chromatic aberrations are more noticeable in Super Resolution and may appear as measurable artifacts when imaging two different fluorochromes. Chromatic aberrations within STORM can be corrected by using “Warp” corrections.

1. **Multi-Color Dyes – imaging order is very important**

In order to reduce photobleaching effects, the longer wavelength dye should be imaged first. If the shorter wavelength dye is imaged first, the high excitation energy of the shorter wavelength laser may also photobleach the longer wavelength dye.

**Fluorescent Probes**

1. **Optimize Fixation**

3% Paraformaldehyde (PFA) + 0.1% Glutaraldehyde

Fixatives should preserve cellular ultrastructure, however some fixation protocols can affect fine cellular structure. Different fixation methods, concentrations may need to be tested to optimize conditions.

1. **Minimize Autofluorescence, Increase Signal-to-Noise**

High background (such as autofluorescence) and/or a weak signal can cause poor signal-to-noise when recording the blinking events, negatively affecting the localization precision by the software. However, once centroids are localized, the background signal will not appear in the final image.

Quenching reagents (0.1% NaBH4) can help reduce the autofluorescence background, improving contrast and increasing the localization precision. In addition, background can also be reduced by using UV-bleached imaging buffers, phenol-free media, and by pre-bleaching the background (1 sec) before acquiring the STORM data set.

1. **Probe Size**

A small probe size will allow probes to be spaced closer together which, in theory, would permit better resolution. However, if the probes are spaced very close together, then a very low Duty Cycle would be required to prevent probe overlap.

Primary + Secondary Antibody = 20nm

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Aptamer (oligonucleotide or peptide) = 2nm

Synthetic Dye – 1nm

1. **Antibody Concentration**

The antibody concentration should be at a level that just saturates the available binding sites. Saturation of the sample with primary and/or secondary antibodies can lead to non-specific binding. Testing antibody concentration (titration) is recommended. Secondary antibodies are typically used in the range of 1:500 to 1:1000 dilution.

If the density of fluorophore is too high, reduce the incubation time, reduce the antibody concentration, and/or mix the secondary antibody with unlabeled secondary antibody.

Check the Density of Label (DoL) for the secondary antibodies. DoL is the number of synthetic dye molecules per antibody. Many secondary antibodies will have 3-8 DoL, but a 1:1 DoL would be preferred for STORM.

If the density of fluorophore is too low (too sparse), then the observation resolution will be poor. Labeling density should be 2-times the intended resolution.

40nm resolution would require 20nm spacing of fluorescent probes in order to meet Nyquist.

If density of probe is too low, then increase antibody concentration, increase incubation time, select different fluorescent probe, try different buffer, make sure buffer is fresh, try different fixation method, increase exposure time, check illumination laser power and illumination angle.

1. **Reduce Non-Specific Labeling**

Non-specific binding of the fluorescent probes to the sample will create artifacts within the final image and can be a significant problem for STORM imaging.

Block with heat-treated, sterile-filtered blocking serum (3% BSA + 0.2% Triton X-100) and thoroughly wash sample with blocking serum at every step to help reduce non-specific binding of antibodies to the sample. Lock secondary antibodies in place with post-staining fixation. Remove residues with Tween 80 wash.

1. **Photoactivatable Fluorescent Proteins**

Fluorescent Proteins by design have a 1:1 label, no non-specific labeling and, for stable lines, can achieve close to 100% protein labeling efficiency. And, since fluorescent proteins are smaller in size than antibody labels, better resolution can be achieved. However, fluorescent proteins also exhibit lower photostability and brightness (600-900 photons/event at best) than organic dyes, resulting in lower localization precision than for organic dyes. And, many fluorescent proteins tend to dimerize, which would also affect resolution.

1. **Recommended Probes**

Far Red: Cy5 or Alexa-647

Red: Alexa-555, Alexa-568, Cy3B

Green: Atto-488

All work well in Glox + 100mM MEA containing buffers.

1. **Use glass-bottom #1.5 coverslip dishes**

**Buffer Concerns**

1. **Jablonski Diagram**

S0 Ground State

S1 Excited State

T1 Triplet State, ISC-InterSystem Crossing, S1 electron unpairs from ground state partner &

reverses spin (spin flip) = forbidden spin transition, low probability.

Off-State lifetime microsecond – second.

T1 can return to S0 in presence of O2.

T1 can also cross to R or RH State in presence of reducing agents (RSH/RS-).

R and RH States can return to S0 in presence of O2 and a 405nm laser pulse.

R State, ISC, in presence of reducing agent.

RH State, ISC, in presence of reducing agent.

Electrons in T1 can return to S0 through the non-radiative transfer of energy from an activated activator to an adjacent reporter residing in a T1 triplet state.

Reduced dyes can also strongly absorb at 405nm, causing electrons in the R and RH states to return to the S0 ground state following UV excitation.

1. **Reducing Agents**

B-mercaptoethanol (BME)

Mercaptoethylamine (MEA)

Reducing agents reduce (protonate) the probe in the T1 state to send the probe to the R or RH states, which are stable radical (unreactive) states. The thiols will bind to the probe, disrupting the pi-conjugated electron cloud, making the probe dark (OFF-State). Reduced probes (the thiol adduct) strongly absorb 405nm light, which removes the thiol, restores the pi-conjugated system, and returns the probe to the S0 ground state.

1. **Oxygen Scavengers**

Glucose Oxidase (GLOX) + Glucose + Catalase

Oxidizing reagents will quench the probe in the T1, R and RH states, returning the probe to the S0 ground state and resulting in a high duty cycle and increased photobleaching.

Glucose oxidase catalyzes the oxidation of glucose by molecular oxygen to create hydrogen peroxide. Catalase then converts the hydrogen peroxide to water and O2.

The reaction by GLOX (which uses two O2) and Catalase (which generates one O2) is the net loss of one O2 molecule.

1. **Buffers**

Any physiological buffers may be used for STORM.

TRIS, which buffers 7.5-9.0, is the most commonly used buffer for STORM. The slightly basic pH helps to counter the drop in pH due to the GLOX activity.

**Camera**

Andor iXon DU897Ultra EMCCD Camera

512x512 pixels

16um pixel resolution (with 100x objective pixel size = 0.16um)

17MHz