

Single-Molecule High-Resolution Colocalization of Single Probes

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Colocalization of fluorescent probes is commonly used in cell biology to discern the proximity of two proteins in the cell. Considering that the resolution limit of optical microscopy is on the order of 250 nm, there has not been a need for high-resolution colocalization techniques. However, with the advent of higher resolution techniques for cell biology and single-molecule biophysics, colocalization must also improve. For diffraction-limited applications, a geometric transformation (i.e., translation, scaling, and rotation) is typically applied to one color channel to align it with the other; however, to achieve high-resolution colocalization, this is not sufficient. Single-molecule high-resolution colocalization (SHREC) of single probes uses the local weighted mean transformation to achieve a colocalization resolution of at least 10 nm. This protocol describes the acquisition of registration data and the analysis required to obtain a high-resolution mapping between imaging channels. The total internal reflection fluorescence microscope (TIRFM) system described is designed to excite and image the fluorescent probes Cy3 and Cy5. Modifications may be required depending on the requirements of the individual study.

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

Fiducial beads (e.g., TransFluoSphere beads 543/620, 100-nm; Molecular Probes/Invitrogen or similar)

MgCl₂

Equipment

Coverslips

LabView routine, custom-made (National Instruments; Austin, TX) (optional; see Step 7B.iii)

MATLAB software program with image processing toolbox (The MathWorks, Natick, MA)

Micropipette tip or microcentrifuge tube

Microscope slides, glass (or quartz, depending on the type of TIRFM used)

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Tape (double-sided; e.g., Scotch, 3M)
 Total internal reflection fluorescence microscope (TIRFM)
 Camera, EMCCD (iXon DV 887 EMCCD; Andor Technology, Belfast, Ireland)
 Emission filters (585/70 and 690/40)
 Iris, adjustable
 Laser (helium neon; 25 mW, 633 nm; JDS Uniphase, San Jose, CA)
 Laser (Nd:Yag; Compass 215 M, 15 mW, 532 nm; Coherent Inc., Santa Clara, CA)
 Lenses ($f = 2.5, 50, 95, 120$, and 500 mm)
 Mirrors (dichroic; 595 LP beam splitter, 625 LP beam splitter and 532–632 nm beam splitter; Chroma Technology Corp., Rockingham, Vermont or similar)
 Mirrors (optical)
 Nanotranslation stage, piezo, closed-loop, two-axis, equipped with capacitive sensors for position measurement (Physik Instrumente, Auburn, MA) (optional; see Steps 7B.i–iii)
 Notch filters (StopLine U-grade [OD 6] 532/17 and 633/25 nm; Semrock Corp., Rochester, NY)
 Objective (Olympus 1.65 NA 100 $\times$ TIRF)
 OD (optical density) filter wheel
 Quarter wave plate (quartz multiorder $\lambda/4$ plate; Newport Corporation, Irvine, CA)
 Razor blades (two, separated by an ~ 1 mm slit, onto which emitted light is focused)
 Translation stage

METHOD

Flow Cell Construction and Loading

1. On a microscope slide, lay down two 1.5-in.-long pieces of double-sided tape with a quarter-inch gap.
2. Ensure that the tape is well adhered to the glass by dragging the edge of another glass microscope slide along the tape.
3. Place a clean coverslip on the tape in the center of the lines of tape. Ensure that the coverslip matches the index of refraction of the microscope's objective.
4. Use a pipette tip or the bottom of a microcentrifuge tube to gently rub the top of the coverslip where it contacts the tape to ensure a good seal has been made.
5. Dilute the fiducial beads in H₂O with 1 mM MgCl₂.
Without the MgCl₂, the beads will not stick to a glass surface. The dilution will depend on the stock solution of the fiducial beads. The beads should end up distributed on the coverslip at a concentration <1 per field-of-view area.
6. Flow 10 μ L of beads into the prepared flow cell.

Fiducial Data Acquisition

7. Collect the fiducial data:
 - A. *With a standard translation stage*
 - i. Move a distribution of beads around the field of view. (The bead density stuck to the coverslip can be higher than one per field of view.)

- ii. Image the field of beads.
- iii. Move the field and reimaged until enough of the field of view has been sampled by the fiducial markers.

B. With a piezo nanostage

- i. Locate a single bead with no neighbors within one to two fields of view.
- ii. Using the piezo nanostage, step the bead through a grid.
- iii. At each position, image the bead in both channels. If the camera is capable of being triggered by a transistor–transistor logic (TTL) pulse, this can be done automatically with a custom-made LabView routine. A LabView routine can control the voltage input going to the piezo stage controller dictating its position, and then a TTL pulse can be sent to the camera signaling it to take an image.

Automating the registration data collection greatly decreases the time it takes to gather the data and reduces the stringency of the photostability requirement of the fiducial marker.

Data Analysis

*Although the registration data can be processed by other means (see **Colocalization of Fluorescent Probes: Accurate and Precise Registration with Nanometer Resolution** [Churchman and Spudich 2012]), the use of MATLAB and its image processing toolbox simplifies matters significantly. Here, we present the MATLAB programming algorithms needed to calculate the local weighted mean (LWM).*

8. Assign locations from each channel to a variable such as “cp_channel1” and “cp_channel2”.

9. Transform the data using the “cp2tform” command:
tform_lwm = cp2tform(cp_channel1, cp_channel2, 'lwm');

10. Calculate the metrics to estimate the error associated with this transformation:

- i. To calculate the fiducial registration error (FRE):
trans_cp_channel2 = tforminv(cp_channel2, tform_lwm);
FRE = sqrt(sum(sum((cp_channel1 –
trans_cp_channel2).^2))/(length(cp_channel1)));
- ii. To calculate the target registration error (TRE):
% find number of control point
number_cp = length(cp_channel1);
% loop through the control points
for i = 1: number_cp
% take out that control point
remain_cp = [1:i-1 i + 1:number_cp];
% calculate the transformation without the ith control
point
tform_lwm = cp2tform(cp_channel1(remain_cp, :),
cp_channel2(remain_cp, :), 'lwm');
% transform the left out control point with the above
% found transformation
trans_cp_channel2(i, :) = tforminv(cp_channel2(i, :),
tform_lwm);
end
TRE = sqrt(sum(sum((cp_channel1 –
trans_cp_channel2).^2))/(length(cp_channel1)));

Applying Registration Mapping to Subsequent Data Using MATLAB

11. For single-molecule data:
point_channel2_trans = tforminv(point_channel2, tform_lwm);
12. For image registration:
image_channel2_trans = imtransform(image_channel2, tform_lwm);

REFERENCE

Churchman LS, Spudich JA. 2012. Colocalization of fluorescent probes: Accurate and precise registration with nanometer resolution. *Cold Spring Harb Protoc* doi: 10.1101/pdb.top067918.