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Imaging Real-Time Gene Expression in Living Systems with Single-Transcript Resolution: Image Analysis of Single mRNA Transcripts

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Abstract

This protocol describes a method for establishing a green fluorescent protein (GFP) calibration curve using dilutions of recombinant GFP and blue fluorescent beads. The total fluorescence intensity (TFI) per mRNA molecule is first calculated by imaging serial dilutions of purified enhanced GFP (eGFP) to determine the TFI within a specific volume. A calibration curve of fluorescence intensity in a given voxel per molecule of GFP is then used to determine the number of GFP molecules in the sample of formaldehyde fixed cells to be imaged. This is followed by a method for detection of single molecules in formaldehyde-fixed and live cells. These cells have been cotransfected with mRNA reporter and MCP-xFP plasmids, where MCP-xFP refers to a fluorescent protein fused to the MS2 capsid protein. It is important to collect micrographs and establish the calibration curve on the same day that the cells are imaged, using the same equipment configuration, camera settings, and image acquisition parameters. Single-molecule measurements using GFP are performed as in Femino et al. (1998, 2003) and Fusco et al. (2003).

MATERIALS

Reagents

Cells to be imaged (cotransfected, as in Imaging Real-Time Gene Expression in Mammalian

Cells with Single-Transcript Resolution, PMID: 21356977)

DAPI (4′,6-diamidine-2-phenylindole dihydrochloride)

Formaldehyde (4%)

Mounting medium (ProLong Gold, Invitrogen)

Phosphate-buffered saline (PBS)

Recombinant monomeric GFP (rGFP; purified; Clontech)

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Equipment

Charge-coupled device (CCD) camera

Coverslips

Flat, heavy object (see Step 3)

Fluorescence microscope with appropriate attachments

Fluorescent microsphere beads (0.2-μm, blue; Invitrogen or Duke Scientific)

Microscope slides

Nail polish

Software package that will calculate the TFI

Stage micrometer (optional; see Step 7)

METHOD

Establishing a GFP Calibration Curve

- 1. Make three serial dilutions of purified rGFP in mounting medium (ProLong Gold) ranging in concentration from 0.1 mg/mL to 0.001 mg/mL.
- 2. Dilute 0.2-μm blue fluorescent microsphere beads 1:1000 to 1:2000 in H_2O. Pipette 5–10 μL of the diluted beads onto a microscope slide. Move the flat edge of a coverslip back and forth to spread a thin layer of the bead solution over the glass surface. Use the same technique to coat a coverslip with 5 μL of diluted beads. (It may take longer to coat the coverslip.) Allow both surfaces to dry. Prepare one set for each dilution of rGFP.

  The microspheres that adhere to the glass surfaces will be used as z-axis markers when determining the size of an imaging voxel.
- 3. For each dilution slide, pipette 5–10 μL of diluted rGFP between the slide and coverslip onto which the blue fluorescent beads were dried. Place a heavy book or flat object on top of the slide and coverslip so that the imaging volume is as thin as possible. Allow the mounting medium to cure overnight in the dark at room temperature under these conditions, or follow the manufacturer's instructions for use. Seal the edges of the coverslip with nail polish and wait until it dries completely before imaging on the microscope.
- 4. Find a field in the sample to image and determine the distance between the two glass surfaces using the blue fluorescent microspheres as upper and lower boundaries in the *z*-axis. This corresponds to the depth of the imaging voxel. To accurately determine this distance, the microscope must have a precise internal focus motor or be fitted with a piezoelectric positioning system. Record the upper and lower positions for each field.

- 5. Once the depth is determined, position the sample halfway between the two glass surfaces so that it sits at the center of the *z*-volume. Switch to the appropriate imaging configuration for GFP and take one image of the field using the same imaging conditions that will be used for the cells. Most software packages have a feature that will calculate the TFI for the field. Record this number.

  If imaging conditions for the cells are not established, determine them before acquiring data for the GFP calibration curve.
- 6. Image several random fields (three or four) following Steps 4 and 5 for each rGFP dilution. The *z*-depth will vary for each field within the slide; define the *z*-depth and the center imaging plane for each field chosen.
- 7. Calculate the pixel size using a stage micrometer or by using this formula: pixel size (microns) = (pixel size of the CCD camera/total magnification) × (binning factor)
- 8. For each field of images:
  - i. Calculate the volume of rGFP solution imaged for each full field with the dimensions calculated in previous steps:
  - Volume (μm^3) = [(# of pixels in *x*-axis) × (pixel size)] × [(# of pixels in *y*-axis) × (pixel size)] × [*z*-depth]
  - ii. Calculate the number of GFP molecules in the imaging volume:

$$\text{no. of GFP molecules} = \frac{[N_A] \times [\text{rGFP concentration } (\text{g}/\mu\text{m}^3)] \times [\text{volume } (\mu\text{m}^3)]}{\text{rGFP molecular weight } (\text{g}/\text{mol})}$$

  - where N_A = Avogadro's number (6.022×10^{23} molecules/mol).
- 9. Determine the average TFI per GFP molecule by plotting the TFI obtained in Step 5 against the number of GFP molecules measured in the imaging volume (obtained from Step 8.ii) for each dilution of rGFP.

  The slope of the line through these points represents the TFI/GFP molecule.

Detection of Single Molecules in Fixed Cells

- 10. Plate cotransfected cells on a coverslip and incubate overnight. Fix the cells in 4% formaldehyde for 10 min.
- 11. Rinse the cells twice for 5 min each time with PBS. Stain the nuclei using DAPI.
- 12. Use ProLong Gold (or the same mounting media used to calculate the GFP calibration curve) to mount the coverslip onto a microscope slide.

Cure the mounting medium for about the same time (overnight at room temperature in the dark) as was used for the calibration sample.

- 13. Image the cells using the same optical and imaging configuration as was used for the calibration sample.

  See Troubleshooting.

- 14. Measure the TFI of each object. For each particle identified in the region of interest (ROI), calculate the number of GFP-MCP particles:

$$\text{no. of GFP molecules} = \frac{\text{TFI of each particle}}{\text{TFI per GFP molecule}}$$

  See Troubleshooting.

Detection of Single Molecules in Live Cells

Once single mRNAs are detectable with good signal-to-noise ratio (SNR) in fixed cells, one can attempt to image them in live cells. Many of the MCP-GFP-containing particles will be moving while a time-lapse movie is being captured.

- 15. Proceed with live cells as follows:
  - i. Capture a time-lapse movie.
  - ii. Choose a frame containing the particle of interest and measure the TFI as described above for fixed cells.
  - iii. As a control, determine a bleaching curve of a fluorescent particle over time so that the rate of photobleaching can be factored into the determination of single-molecule fluorescence.

TROUBLESHOOTING

Problem

It is difficult to identify mRNA particles above background.

[Steps 13, 14]

Solution

Consider the following:

- 1. Follow the steps in the Troubleshooting section of Imaging Real-Time Gene Expression in Mammalian Cells with Single-Transcript Resolution (PMID: 21356977) regarding poor SNR to change the microscope or camera configurations and thus increase the SNR.
- 2. Use a deconvolution software program (such as Huygens, SVI) to process a *z*-series stack of the cell. Collect *z*-series image stacks by

stepping through the entire height of the cell in 0.2-μm increments. A built-in *z*-axis motor is required to step through the sample to obtain multiple *xy*-plane images. If deconvolved image planes are used to measure the TFI of particles, divide the TFI of the plane by the number of planes in the imaging volume.

- 3. Define particles using analysis software and sum their TFIs from a *z*-stack. The background intensity should not sum to the same extent as the fluorescent object. Thus, the particle should emerge with a greater SNR. Divide the sum of the TFIs by the number of planes to give an average TFI value. Divide this number by the TFI per GFP molecule to determine the number of GFP molecules contained in the particle.

DISCUSSION

Using the methods described here, Fusco et al. (2003) determined that one mRNA particle engineered with 24 MBSs binds an average of 33 molecules of MCP-GFP. This value may be an underestimate of GFP-MCPs bound, because a maximum of 48 GFP molecules can theoretically bind to 24 MBSs as 24 MCP-GFP dimers.

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References

Femino AM, Fay FS, Fogarty K, Singer RH. Visualization of single RNA transcripts in situ. Science. 1998; 280:585–590. [PubMed: 9554849]

Femino AM, Fogarty K, Lifshitz LM, Carrington W, Singer RH. Visualization of single molecules of mRNA in situ. Methods Enzymol. 2003; 361:245–304. [PubMed: 12624916]

Fusco D, Accornero N, Lavoie B, Shenoy SM, Blanchard JM, Singer RH, Bertrand E. Single mRNA molecules demonstrate probabilistic movement in living mammalian cells. Curr Biol. 2003; 13:161–167. [PubMed: 12546792]

Shav-Tal, Y.; Shenoy, SM.; Singer, RH. Live cell imaging: A laboratory manual, Visualization and quantification of single RNA molecules in living cells. Goldman, RD.; Spector, DL., editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor; New York: 2005. p. 603-615.