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Imaging Real-Time Gene Expression in Mammalian Cells with Single-Transcript Resolution

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Abstract

The MS2 system provides optimal sensitivity for single-molecule detection in cells. It requires two genetically encoded moieties: a reporter mRNA that contains MS2 binding site (MBS) stem loops and a fluorescent MS2 coat protein (MCP-xFP) that binds to the stem loops with high affinity, thus tagging the mRNA within the cell. This protocol describes transfection of COS-7 cells with reporter RNA (e.g., pRSV-Z-24 MBS-β-actin) and MCP-xFP (e.g., pPolII-MCP-GFP-NLS) plasmids using calcium phosphate precipitation. The reporter mRNA plasmid must be co-transfected with the MCP-xFP-NLS plasmid for simultaneous expression in a cell. The unbound MCP-xFP-NLS is sequestered in the nucleus, leaving only the MCP-xFP-NLS that is bound to the reporter mRNA in the cytoplasm. This provides a high signal-to-noise ratio (SNR) that permits detection of single mRNA molecules. The Delta T Imaging System is used for image acquisition of fluorescent particles in the cells.

MATERIALS

Reagents

Calcium phosphate transfection kit (Invitrogen)

Carrier DNA (e.g., sheared salmon sperm DNA)

COS-7 cells, maintained in a 37°C tissue culture incubator with 5% CO_2

COS-7 cells are a transformed monkey kidney fibroblast cell line (ATCC CRL-1651). They are relatively flat, which helps to minimize the amount of out-of-focus light that could interfere with single-molecule detection.

Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS)

This is used as medium for maintaining the COS-7 cells. Alternatively, Leibovitz’s L-15 medium can be used, especially for live-cell imaging (see Step 4).

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Extracellular matrix (ECM) molecules, such as fibronectin, collagen, or Matrigel (BD Biosciences) (optional; see Step 1)

MCP-xFP plasmid (e.g., pPolII-MCP-GFP-NLS; see Table 1)

Mineral oil

Reporter RNA (e.g., pRSV-Z-24 MBS-β-actin)

Equipment

Delta T Culture Dish System (Bioptechs, Inc.)

This technology is specifically designed to maintain precise temperature control of the cells during live-cell imaging. The temperature is tightly regulated by a rapid thermal response feedback loop system that consists of a heated stage insert specifically designed for Delta T dishes and the temperature controller unit (#6 at the top right of Fig. 1*). We use the open dish system for experiments designed for short periods (1 h). For imaging with a closed chamber system, we suggest using the Bioptechs Focht Chamber System 2 (FCS2).*

Electron-multiplying charge-coupled device (EMCCD) camera

Fluorescence microscope with appropriate attachments (see Fig. 1)

Glass coverslips or glass-bottom dishes suitable for live-cell imaging (e.g., Delta T dishes, Bioptechs, Inc.)

Delta T dishes are an open dish imaging system that can be used with the Delta temperature controller. Delta T dishes made with black opaque polystyrene are useful to restrict ambient light from the imaging chamber. These dishes are also available without the temperature-conducting thin film coating on the bottom of the glass imaging surface if temperature control is not needed.

Incubator, preset to 37°C and 5% CO_2

METHOD

Transfection of COS-7 Cells

- 1. Plate COS-7 cells at a density of ~30%–50% confluence in DMEM containing 10% FBS on glass coverslips or in glass-bottom dishes that are suitable for live-cell imaging. Incubate overnight at 37°C in a 5% CO_2 atmosphere.

  The application of extracellular matrix (ECM) molecules such as fibronectin, collagen, or Matrigel to the surface of the glass may be required to facilitate cell adhesion and imaging. Coat the glass surface as needed with appropriate ECM prior to plating the cells.

- 2. The following day, transfect the reporter plasmid with the MCP-xFP plasmid using a calcium phosphate transfection kit. Following the

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manufacturer's instructions, add the precipitate dropwise to the cells. Incubate the cells overnight.

Determine optimal transfection efficiency by varying the amount and ratio of reporter RNA to MCP-xFP plasmid. Begin by using 5 μg of each plasmid and 5 μg of a nonspecific carrier DNA, such as sheared salmon sperm DNA. The addition of a carrier DNA greatly improves the efficiency of transfection.

Image Acquisition Using the Delta T Imaging System

- 3. Prior to imaging cells, turn on all electronic equipment, including the light source, temperature controllers/objective heaters, environmental controls, camera, and microscope. Allow the imaging system to stabilize for at least 1 h. This will help minimize noise and *z*-drift during imaging.
- 4. Place the Delta T dishes into the Delta T stage adapter set for 37°C. The objective should be fitted with an objective heater (see #6 at the bottom right of Fig. 1). When imaging with an open dish, prevent changes in pH or osmolality by covering the media with mineral oil if a non-CO_2-based media formulation is used. As an alternative, Leibovitz's L-15, which uses a HEPES-based rather than bicarbonate-based buffering formulation, can be used for pH control, but mineral oil is still recommended to prevent evaporation of the medium. This is especially critical when imaging at 37°C.
- The microscope itself is not encased in an environmental chamber or otherwise temperature controlled.

  If yeast is being imaged, use an objective with high magnification and with an NA of 1.4 or higher. Use the media conditions described in Imaging Real-Time Gene Expression in Living Yeast (PMID: 21356978).
- 5. Once the cells are properly temperature controlled and the imaging system has equilibrated, scan the dish for fluorescing cells.
- Many of the positive transfectants will have relatively bright nuclei, due to an excess of MCP-xFP-NLS protein. Therefore, minimal excitation light is needed to identify these cells. Some cytoplasmic mRNA granules may be seen with low light. However, visualization may require saturation of the nuclear MCP-xFP signal.
- 6. Capture a time-lapse movie of a region of the cytoplasm to distinguish moving from stationary particles, using the information listed here:
  - i. mRNA particles in mammalian cells move at speeds up to 1 μm/sec. Use an EMCCD camera with a frame rate of at least 7–9 frames/sec and exposure times of 25–100 msec.

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- ii. Increased incident light may be required to illuminate the fluorescent proteins to achieve a good SNR at low exposure times, but keep in mind that higher light intensities can cause more free radical damage and oxidative stress to the cell.
- iii. Optimize exposure time, light intensity, and viability of the cell before attempting to increase image acquisition speed.
- iv. Depending on the expected velocities of mRNAs, 4D image stacks can be captured and later deconvolved or reconstructed. Due to the high speed of many cytoplasmic processes in mammalian cells, taking *z*-series stacks over time may only be beneficial if the purpose is to specifically track particles for spatial information because the temporal sensitivity may be lost.

  See Troubleshooting.

- 7. Analyze the images as described in Imaging Real-Time Gene Expression in Living Systems with Single-Transcript Resolution: Image Analysis of Single mRNA Transcripts (PMID: 21356979) and Imaging Real-Time Gene Expression in Living Systems with Single-Transcript Resolution: Single mRNA Particle Tracking with ImageJ-Based Analysis (PMID: 21356980).

TROUBLESHOOTING

Problem: No transfected cells are observed or transfection efficiency is low.

[Step 6]

Solution: Try one or more of the following:

- 1. Some mRNA particles are difficult to observe under live-cell imaging conditions, especially if they are motile. Fix cells using 4% formaldehyde (20 min followed by two phosphate-buffered saline [PBS] washes), counterstain nuclei with DAPI, and scan the culture for positive transfectants.
- 2. Use a different transfection method. In addition to calcium phosphate coprecipitation, the following methods have been used successfully to cotransfect MCP-xFP and reporter mRNAs into cells: Electroporation (Shav-Tal et al. 2005), nucleofection (Amaxa Inc.), FuGENE 6 (Roche Diagnostics Corp.), Lipofectamine 2000 (Invitrogen), and lentiviral infection have been used to transfect a range of mammalian cell types.
- 3. Ensure that the plasmid DNA is not degraded and not contaminated with protein. The plasmid DNA should not run as a smear on an agarose

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gel and the A_{260}/A_{280} nm wavelength ratio should be 1.8–2.0. Phenol/chloroform-extract, if necessary, to eliminate protein contaminants from the plasmid DNA.

- 4. Cotransfect a known fluorescent protein as a marker for transfection (other than the color of the MCP-xFP). This will help identify positively transfected cells.
- 5. Confirm that the promoters driving expression of the transgenes will express in your cells.

Problem: Cell viability is low.

[Step 6]

Solution: Consider these suggestions:

- 1. Check the quality of the plasmid DNA. Endotoxin contamination may decrease cell viability. Use an endotoxin-free plasmid DNA purification kit (Qiagen), which can also help increase transfection efficiency.
- 2. The transfection reagent may be toxic to the cells. Always perform a control transfection using the transfection reagent or delivery system without plasmid DNA.
- 3. High levels of MCP-xFP expression can be toxic to some cells. To lower the expression levels, try the following:
  - Decrease the amount of time that the cell expresses the plasmids from overnight to 4–6 h.
  - Transfect less MCP-xFP plasmid DNA without changing the amount of reporter plasmid.

Problem: No RNA particles are observed.

[Step 6]

Solution: Consider these suggestions:

- 1. Screen many transfected cells. Doubly transfected cells are sometimes difficult to find.
- 2. If the reporter construct does not encode for a fluorescent protein product as a convenient transfection marker, fix the cells and use fluorescence in situ hybridization (FISH) to verify that the reporter mRNA is expressed. The best FISH results are obtained using Cy3-labeled probes against the MS2 nonbinding site region (MNBS), which is located between adjacent MS2 binding site stem-loops (see http://www.singerlab.org/protocols/ for probe design, labeling and FISH protocols for both mammalian cells and yeast). The MNBS probe will identify reporter mRNA that has MCP-xFP fusion proteins bound to the

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stem-loops and can serve as a way to verify and quantify single mRNA transcripts (see Imaging Real-Time Gene Expression in Living Systems with Single-Transcript Resolution: Image Analysis of Single mRNA Transcripts, PMID: 21356979). The FISH signal will be very strong if multiple binding sites in the reporter mRNA are used. Alternatively, use fluorescent probes directed against parts of the reporter mRNA that do not include the MS2 binding sites. However, the resulting signal may be weaker and may create false positive results by recognizing endogenous mRNAs in addition to the exogenously expressed mRNA.

- 3. MS2 binding sites can be lost during plasmid DNA amplification in *E. coli*. Use MAX Efficiency Stbl Cells (Invitrogen) for cloning the MS2 binding sites. Always verify that the amplified DNA retains the 24 MBSs by performing a restriction digest and separating the DNA fragments on an appropriate percentage agarose gel. Twenty-four MBSs should run around 1.4 kb.

Problem: There is high background fluorescence.

[Step 6]

Solution: Consider these suggestions:

- 1. If the expression level of MCP-xFP is high, it may be difficult to distinguish particles above the high xFP background. Transfect less of the MCP-xFP plasmid DNA or image at an earlier time post-transfection.
- 2. Try using the MCP-xFP-NLS fusion protein if you are only interested in measuring cytoplasmic mRNA particles. If there is a high nuclear MCP-xFP-NLS signal and no cytoplasmic xFP particles, cotransfect more of the reporter mRNA plasmid DNA, so that the MCP-xFP can bind to the reporter and be exported from the nucleus to the cytoplasm. If only the nucleus is being examined, use the MCP-xFP without the NLS.

Problem: The SNR is poor.

[Step 6]

Solution: Try one or more of the following:

- 1. Transfect more MCP-xFP plasmid DNA.
- 2. Increase the gain of the camera.
- 3. Increase the exposure time and/or the intensity of the excitation light source. Keep in mind that this may adversely affect the health and viability of cells during live-cell imaging and can decrease the time resolution of time-lapse movies. This can affect the accuracy of measuring particle movements.

- 4. We have successfully used an objective-based TIRF microscope to improve the SNR of MCP-xFP particles in live cells. Owing to the principle of TIRF, only the most ventral 150–200 nm of the cell is visible. Particles may move in and out of the evanescent field, which may complicate single-particle tracking. However, for very thin cells, this may prove to be very advantageous.

Problem: Cells/particles do not remain in focus during imaging.

[Step 6]

Solution: Try one or more of the following:

- 1. Focus drift may result from an unstable stage, or more likely, from an objective that is unable to maintain stable axial positioning. Carefully control the temperature of the imaging system (especially the objective and other metal components of the microscope base) by equilibrating the system within the room or an environmental chamber for at least 1 h prior to imaging. This includes turning on all of the temperature-controller units that will be used to maintain temperature.
- 2. Use a ZDC/autofocus correction system. This solution requires installation of a piezoelectric positioning device and a far-red laser source for position sensitive detection. Olympus sells their motorized IX81 inverted microscope stand with a ZDC (model IX81-ZDC zero drift autofocus system) or it can be retrofitted onto existing stands. The ZDC uses a 735-nm laser diode to accurately find the specimen-glass coverslip interface and reliably returns to this position during imaging. Using a similar approach to sensing the coverslip-sample interface, Applied Scientific Instrumentation sells the CRIFF-2000 external autofocus device that fits most inverted microscope stands but which requires either a TIRF objective or reflective-coated coverslips.
- 3. Take *z*-series image stacks to capture particle fluctuations within the *z*-axis. This helps track the particles and allows for 3D reconstruction of particle movements using postprocessing software. However, it will result in longer acquisition times (since more images are taken), which in turn leads to less accurate velocity estimates and an increased likelihood of phototoxicity.

Problem: Photobleaching occurs.

[Step 6]

Solution: Try one or more of the following:

- 1. Include enzymatic oxygen and oxygen free-radical-scavenging systems in the imaging medium. OxyFluor (Oxyrase, Inc.) is designed specifically to reduce photobleaching of fluorescent dyes and is compatible with live-

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cell imaging (Waterman-Storer et al. 1993; Mikhailov and Gundersen 1995;Waterman-Storer et al. 2000). Ascorbic acid (vitamin C) at 2 mg/mL and Trolox, a cell-permeable, water-soluble vitamin E derivative, at 200 μM (Salgo et al. 1995; Salgo and Pryor 1996), can also be used as anti-fade reagents. For fixed cells, try the glucose oxidase-catalase enzyme system (Park et al. 2004).

- 2. Decrease the excitation light intensity by placing neutral density filters in the optical light path between the light source and the objective. This may require increasing the exposure time for each frame, increasing the camera gain, and/or increasing binning to capture more of the emitted fluorescence light.
- 3. Decrease the exposure time of each frame if there is no way to attenuate the excitation light intensity.

DISCUSSION

The cytomegalovirus (CMV) promoter and the Rous Sarcoma Virus (RSV) long terminal repeat provide strong expression of the reporter mRNA in a wide range of cell types. To image single mRNA particles in COS-7 cells, Fusco et. al. (2003) used the RSV promoter to drive expression of a reporter gene in which the 24-MBS cassette was inserted between the LacZ coding sequence and the 3′UTR of β actin (pRSV-Z-MS2x24-β actin). An SV40 polyadenylation site was added downstream of the β actin 3′UTR for transcriptional termination.

The level of expression of the MCP-xFP must be carefully determined to resolve single molecules, because overexpression can lead to high fluorescence background due to unbound MCP-xFP. The large subunit of RNA polymerase II (polII) and RSV promoters give acceptable levels of MCP-xFP in primary fibroblasts and COS-7 cells, respectively. However, the CMV promoter caused high background and cell toxicity. Targeting unbound MCP-xFPs to the nucleus is one way to titrate the levels of MCP-xFP in the cytoplasm. The detection of single mRNA molecules in the cytoplasm improved when the NLS from the simian virus 40 (SV40) large T antigen was cloned in frame following GFP (pMS2-GFP-NLS) (Bertrand et al. 1998; Fusco et al. 2003)

Cells are extremely sensitive to their environment and can respond to changes by eliciting a stress response or apoptosing. The major environmental conditions that can affect cell viability are: (1) temperature, (2) pH, (3) osmolality, (4) free radical production, and (5) phototoxicity. For an excellent discussion of how to optimize imaging of live cells, see Swedlow et al. (2005). Obtaining high-quality images is essential for subsequent image analysis. The goal is to acquire images with wide dynamic range, high SNR, and high time resolution to accurately track moving particles while keeping the cell alive. Usually, one of these parameters will need to be sacrificed to optimize others. Because our goal is to detect single particles, we detail image acquisition that favors high signal-to-noise detection with moderate time resolution. Under these circumstances, the most crucial component of the imaging system is the camera. EMCCD cameras offer high sensitivity at fast frame rates.

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Faster acquisition rates can be tested after one has clearly established reliable single molecule sensitivity (see Imaging Real-Time Gene Expression in Living Systems with Single-Transcript Resolution: Image Analysis of Single mRNA Transcripts, PMID: 21356979).

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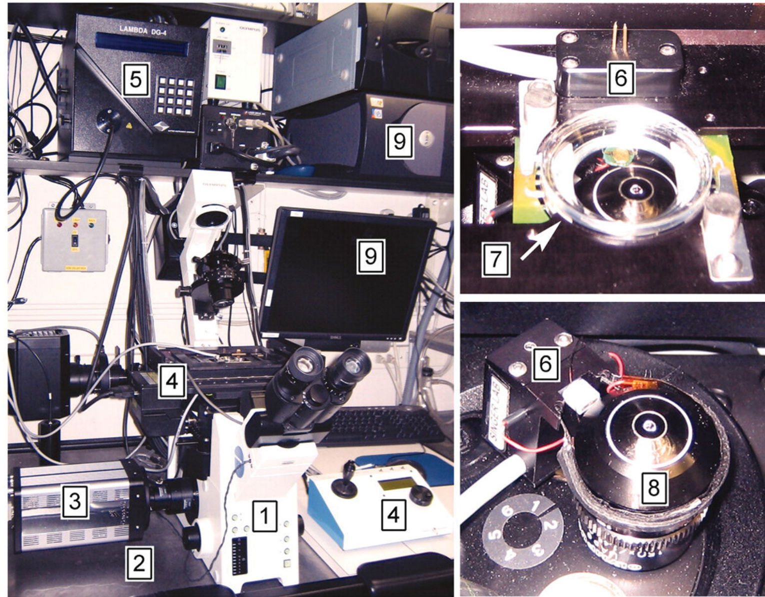

Figure 1.

Illustration of a microscope setup designed for single-molecule fluorescence live-cell imaging. *(Left)* A fully automated microscope system includes a base stand with a (1) built-in autofocus system, (2) vibration isolation table, (3) an ultrasensitive and fast camera, (4) *x*-, *y*-, and *z*-stage positioning, (5) tunable light source, and (9) a multifunctional software package that controls all hardware components and data acquisition. (*Top right*) The Delta T dish system (Bioptechs, Inc.) shows the glass bottom dish (*7*) securely positioned in its temperature-controlled stage mount (6). (*Bottom right*) A high-magnification and NA objective (8) is shown with an objective heater mounted to it (6).

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Table 1

Expression vectors for MCP-xFP fusion proteins for use in mammalian cells

Vector	MCP-fusion	Promoter	Selection	Reference
pMS2-GFP-NLS	MCP-GFP-NLS	Pol II	Kan/Neo	Fusco et al. (2003)
GFP-MS2-1xNLS	GFP-MCP-1xNLS	CMV	Kan/Neo	Dynes and Steward (2007)
bNLS-GFP-MS2	bNLS-GFP-MCP[a]	CMV	Kan/Neo	Dynes and Steward (2007)
K89	GFP-NLS-MCP	CMV	Kan/Neo	Bi et al. (2003)

[a]Bitartate NLS from *Xenopus* nucleoplasmin protein