Video Article

Visualizing and Tracking Endogenous MRNAs in Live *Drosophila Melanogaster* Egg Chambers

Irina E. Catrina[1], Livia V. Bayer[1,2], Omar S. Omar[1,2], Diana P. Bratu[1,2]

[1]Biological Sciences Department Hunter College, City University of New York

[2]Program in Molecular, Cellular, and Developmental Biology, Graduate Center, City University of New York

Correspondence to: Irina E. Catrina at icatrina@hunter.cuny.edu, Diana P. Bratu at bratu@genectr.hunter.cuny.edu

Abstract

Fluorescence-based imaging techniques, in combination with developments in light microscopy, have revolutionized how cell biologists conduct live cell imaging studies. Methods for detecting RNAs have expanded greatly since seminal studies linked site-specific mRNA localization to gene expression regulation. Dynamic mRNA processes can now be visualized via approaches that detect mRNAs, coupled with microscopy set-ups that are fast enough to capture the dynamic range of molecular behavior. The molecular beacon technology is a hybridization-based approach capable of direct detection of endogenous transcripts in living cells. Molecular beacons are hairpin-shaped, internally quenched, single-nucleotide discriminating nucleic acid probes, which fluoresce only upon hybridization to a unique target sequence. When coupled with advanced fluorescence microscopy and high-resolution imaging, they enable one to perform spatial and temporal tracking of intracellular movement of mRNAs. Although this technology is the only method capable of detecting endogenous transcripts, cell biologists have not yet fully embraced this technology due to difficulties in designing such probes for live cell imaging. A new software application, *PinMol*, allows for enhanced and rapid design of probes best suited to efficiently hybridize to mRNA target regions within a living cell. In addition, high-resolution, real-time image acquisition and current, open source image analysis software allow for a refined data output, leading to a finer evaluation of the complexity underlying the dynamic processes involved in the mRNA's life cycle.

Here we present a comprehensive protocol for designing and delivering molecular beacons into *Drosophila melanogaster* egg chambers. Direct and highly specific detection and visualization of endogenous maternal mRNAs is performed via spinning disc confocal microscopy. Imaging data is processed and analyzed using object detection and tracking in Icy software to obtain details about the dynamic movement of mRNAs, which are transported and localized to specialized regions within the oocyte.

Introduction

Cell biology studies that visualize dynamic events with spatial and temporal resolution have been made possible by the development of fluorescence-based live cell imaging techniques. Presently, *in vivo* mRNA visualization is achieved via technologies that are based on RNA aptamer-protein interactions, RNA aptamer-induced fluorescence of organic dyes and nucleic acid probe annealing[1,2,3]. They all offer high specificity, sensitivity and signal-to-background ratio. However, RNA aptamer-centered approaches require extensive genetic manipulation, where a transgene is engineered to express an RNA with artificial structural motifs that are required for protein or organic dye binding. For example, the MS2/MCP system requires the co-expression of a transgene expressing an RNA construct containing multiple tandem repeats of the binding sequence for the bacteriophage MS2 coat protein (MCP), and another transgene encoding a fluorescent protein fused to MCP[4,5]. The addition of such secondary structural motifs to the RNA, along with a bulky fluorescently tagged protein, has raised concerns that native RNA processes may be affected[6]. A technology that addresses this concern and offers additional unique advantages is the nucleic acid-based approach, molecular beacons (MBs). MBs allow for the multiplex detection of endogenous mRNAs, discrimination of single nucleotide variations, and fast kinetics of hybridization with target mRNA[7,8]. MBs are oligonucleotide probes that remain in a quenched hairpin fold prior to undergoing a fluorogenic conformational change once they hybridize to their targets (**Figure 1C**)[9]. Several groups have had success in using MBs to detect both non-coding RNAs (microRNAs and lncRNAs)[10,11,12,13], RNA retroviruses[14] and dynamic DNA-protein interactions[15]. They have been successfully employed for imaging in various organisms and tissues, such as zebrafish embryos[16], neurons[13], tumor tissue[17], differentiating cardiomyocytes[18], and *Salmonella*[19].

Here we describe the design, delivery and detection approach for endogenous mRNAs in living *D. melanogaster* egg chambers coupled with a microscopy set-up that is fast enough to capture the dynamic range of active molecular transport. The *D. melanogaster* egg chamber has served as an ideal multicellular model system for a wide range of developmental studies, from early germline stem cell division and maternal gene expression to the generation of segmental body plan[20,21]. Egg chambers are easily isolated, large and translucent, and able to withstand hours of *ex vivo* analysis, making them highly amenable to imaging experiments. Much work has focused on the asymmetric localization of maternal transcripts to discrete subcellular regions prior to being actively translated. In particular, *oskar* mRNA localization and its subsequent translation at the oocyte's posterior pole must occur in a tightly regulated manner to avoid a lethal bicaudal embryo phenotype[22]. *oskar* mRNA is transcribed in the 15 germline cells, called nurse cells, and actively transported through cytoplasmic bridges, called ring canals, into the oocyte, the germline cell that becomes the mature egg and is ultimately fertilized (**Figure 1A**). The considerable amount of information already available

regarding the dynamic recruitment and exchange of protein factors to and from *oskar* mRNP, along with its long-range intracellular travel, make *oskar* a preferred candidate to study the many processes of the mRNA life cycle. MBs have been instrumental in revealing details about the process of mRNA localization and deciphering the regulation and function of protein factors that control mRNA transport during *Drosophila* oogenesis. In particular, by microinjecting MBs into nurse cells and performing live cell imaging experiments, the tracking of endogenous mRNAs is possible[8,23].

The roadmap presented here offers the steps of a complete process, from carrying out a live cell imaging experiment using MBs, acquiring imaging data, to performing data analysis to track endogenous mRNA in its native cellular environment. The steps can be modified and further optimized to meet the needs of researchers working with other tissues/cell types within their own lab setting.

Protocol

1. Design of MBs for Live Cell Imaging

1. Fold the target RNA sequence to predict the mRNA target's secondary structure using the "RNA form" from the *mfold* server (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form).
    1. Paste/upload the target sequence in FASTA format, select 5 or 10% sub-optimality (structures with a free energy of folding within 5 or 10% of the MFE value, respectively), and adjust the maximum number of computed foldings accordingly (*e.g.* larger for 10% sub-optimality).
    Note: Inclusion of sub-optimal secondary structures when designing MBs allows for the identification of regions within the target mRNA that may be more flexible or more rigid than as predicted for the minimum free energy (MFE) structure alone, which improves the overall design of MBs suited for live cell imaging.
    2. Select an "immediate job" for mRNA targets of 800 nucleotides (nt), or a "batch job" for mRNA lengths between 801 and 8,000 nt. Save the "ss-count" file as simple text file.
2. Use the "ss-count" file obtained in step 1.1 as input for the *PinMol* program (https://bratulab.wordpress.com/software/) with the desired parameters, to design several MBs for the mRNA target (see tutorials describing usage of *PinMol* program[24] at https://bratulab.wordpress.com/tutorial-pinmol-mac/).
    1. Determine the specificity of selected MBs by performing BLAST analysis: use "blastn" with the appropriate database (*e.g.* for *oskar* mRNA-specific MBs use the "refseq-rna" database and the *Drosophila melanogaster* organism).
    2. Identify any tissue-specific expression of mRNA target (*e.g.* for *oskar* mRNA Flybase> High-Throughput Expression Data> FlyAtlas Anatomy Microarray or modENCODE Anatomy RNA-Seq; http://flybase.org/reports/FBgn0003015) and compare with any positive BLAST hits. Eliminate probes that show > 50% cross-homology with other mRNAs that are also expressed in the tissue/cell of interest.
3. Select the fluorophore and quencher pair appropriate for the microscopy set-up available to perform live cell imaging (*e.g.* Cy5/BHQ2)[25].

2. MB synthesis, purification and characterization

1. Use in-house synthesis and purification as previously described[7], or services from commercial providers, to synthesize and purify one to five MBs (see above note), using the following labeling scheme: [5'(Fluorophore)-(C3 or C6 linker)-(2'-O-methyl MB sequence)-(Quencher)3']. Purify MBs using reverse-phase HPLC, in house or using the services of the commercial provider.
Note: The phosphoramidites used for automated probe synthesis must have the 2'-O-methyl ribonucleotide modification. One can also use chimeras of alternating locked-nucleic acid (LNA) and 2'-O-methyl modifications to increase the stability of a hybrid between a shorter MB and its target mRNA[26].
2. Synthesize DNA oligonucleotides that match the sequence of the targeted RNA region and thus are complementary to the probe region of MBs, for use in *in vitro* characterization (see steps 2.3 to 2.5; above note). Maximize hybridization of the MB with the DNA-oligonucleotide target mimic, by including on each end of the DNA target four additional nucleotides, as found in the target mRNA sequence.
Note: A more rigorous characterization of the MB's efficiency to detect the targeted sequence can be performed using *in vitro* synthesized RNA targets instead of complementary DNA oligonucleotides[8].
3. Perform thermal denaturation of the MB alone, measure its melting temperature (Tm), and confirm that the MB assumes the desired hairpin shape at physiological temperature. We have observed Tm values between 60 and 90 °C.
4. Perform thermal denaturation of the MB in the presence of the DNA oligonucleotide target and measure the MB:DNA target hybrid's Tm, as previously described[7]. A Tm between 55 and 60 °C is desired for the MB:DNA hybrid.
5. Perform *in vitro* hybridization reactions with the corresponding DNA oligonucleotide target, and determine the efficiency of MB:DNA hybrid formation at physiological temperature, as previously described[7]. Fast hybridization kinetics with the DNA target mimic is desired, however MBs that do not show high hybridization efficiency with DNA targets may have a better performance with the target mRNA *in vitro* and/or *in vivo*.

3. Dissection and Preparation of Individual Egg Chambers for Microinjection

1. Feed newly hatched, mated females for 2-3 days with fresh yeast paste.
2. Anesthetize flies on a CO_2 pad and, using fine tweezers (Dumont #5), transfer 1-2 females into a drop of Halocarbon oil 700 on a glass cover slip.
3. Using a pair of tweezers, orient the fly with the dorsal side up under a stereomicroscope. Dissect the female abdomen by making a small incision at the posterior end and gently squeeze the pair of ovaries into the oil.
4. Explant the ovaries onto an oil drop on a new coverslip. Gently hold one ovary with one tweezer while pinching off the youngest stages of the ovariole with the other tweezer. *oskar* mRNA is actively localized at and after mid-oogenesis (stages > 7), and younger egg chambers

(stages < 7) are more difficult to inject and do not survive as long. Slowly drag on the cover slip (with a downward movement) until individual ovarioles or egg chambers are isolated and aligned vertically. Further separate single egg chambers by displacing the unwanted stages from the ovariole egg chain.
Note: Ensure that individually teased egg chambers do not float in the oil, and that they adhere to the cover slip. This is important for both successful microinjection and image acquisition.

4. Microinjection of MBs into the Nurse Cells of Egg Chambers

1. Prepare the MB solution, using one molecular beacon (*e.g.* osk2216Cy5), or a mix of two MBs that target different mRNAs and which are labeled with spectrally distinct fluorophores (*e.g.* osk2216Cy5 and drongo1111Cy3). Use a concentration of 200-300 ng/µL each MB in HybBuffer (50 mM Tris-HCl - pH 7.5, 1.5 mM MgCl$_2$ and 100 mM NaCl). For a cocktail of four MBs labeled with the same fluorophore that are targeting the same mRNA at 200 ng/µL each in HybBuffer (*e.g.* osk82, osk1236, osk2216). Spin down the MB solution immediately prior to loading the needle for microinjection.
2. Select the objective. A 40X oil objective is recommended for finding an appropriate egg chamber and for performing microinjection.
3. Mount the coverslip with dissected egg chamber onto the microscope stage. Bring up the objective in the focus position and identify an egg chamber at a mid-to-late developmental stage, that is properly oriented for microinjection (*i.e.,* with the AàP axis perpendicular to the needle tip to allow for easy injection within a nurse cell proximal to the oocyte).
4. Load a needle (commercial or prepared in house[27]) with ~1 µL MB solution (*see* step 4.1) and connect it to the microinjector. For microinjections in *D. melanogaster* egg chambers, orient the needle (see **Table of Materials**) at an angle < 45° to the microscope stage (*e.g.* 30°) to avoid puncturing several nurse cells.
5. Set-up the injector with injection pressure of 500-1,000 hPa and compensation pressure of 100-250 hPa (see **Table of Materials**).
6. Slowly move the stage to bring in the field of view an area of the oil drop void of egg chambers.
7. Using the micromanipulator joystick, gently lower the needle into the oil drop and bring its tip into focus towards the periphery of the field of view.
8. Perform a 'clean' function to remove the air from the tip of needle and to ensure that there is flow from the needle.
9. Bring the needle to the home position and focus on the egg chamber to be microinjected, then bring the needle back into focus and position it near the edge of the egg chamber.
10. Perform a fine adjustment of the objective's Z-position such that the membrane separating the follicle cells from nurse cells is in focus.
11. Insert the needle into a nurse cell and perform injection for 2-5 s.
12. Gently remove the needle and retract it to the home position.
13. Change the objective to the desired magnification for image acquisition (60-63X or 100X), focus on the egg chamber, and begin acquisition.

5. Acquisition of Data Using a Spinning Disc Confocal Microscope Setup

Note: See **Table of Materials** for our specific setup.

1. Set up acquisition protocol to record an XYZCt stack of 8-16-bit images (XYZ = volume, C = channel, t = time).
2. Select laser lines for the desired channels (*e.g.* 641 nm laser for Cy5 and 491 nm for GFP) and acquire the channels sequentially: first the fluorescence signal in each channel and then change the Z position, to allow for proper colocalization analysis.
3. Select the Z step (*e.g.* 0.3 µm), and the top and bottom Z limits (*e.g.* -2 µm to 2 µm).
4. Input the acquisition time and sampling rate (*e.g.* every 15-30 s for up to 1 h).
5. Initiate acquisition.

6. Processing, Data Analysis to Obtain Tracking and Colocalization Information, and Preparation of Video Files

1. Image Processing
    1. Download, unpack, and open Icy, an open community platform for bioimage informatics (http://icy.bioimageanalysis.org/)
    2. Open the XYZCt stack acquired in step 5: Image/Sequence >File>Open.
    3. Convert stack to ImageJ: ImageJ> Tools> Convert to IJ, have Detached Mode ON.
    4. Make a substack (a selection of a range of Z steps and time points to be further analyzed): ImageJ>Image>Stacks>Tools>Make Substack…; select the desired channels, Z-steps and time points.
    5. Save substack as TIFF file: ImageJ>File>Save As>Tiff…; use this file for subsequent steps.
    6. Split channels: ImageJ>Image>Color>Split Channels.
    7. Subtract background either using a background stack: ImageJ>Process>Image Calculator…, or using the Rolling ball option: ImageJ>Process>Subtract Background…, select the Rolling ball radius. Preview the image for the radius selected before selecting "Accept".
    Note: Background signal will mainly arise from improper quenching of the fluorophore. The signal:background ratio (S:B) is often used as an indicator for an MB's "brightness", and it is measured from *in vitro* hybridization experiments of the MB and DNA target oligonucleotide. For example, MBs osk1236 and osk2216 have an S:B of ~81 and ~120, respectively.
    8. Adjust the brightness and contrast for each channel: ImageJ>Image>Adjust>Brightness/Contrast, select Apply.
    9. Save each channel as a separate TIFF file: ImageJ>File>Save As>Tiff....
    10. Merge the two channels: ImageJ>Image>Color>Merge Channels…; select the channels. Save the new stack as a new TIFF file (*see* step 6.1.8).

2. Spot Detection and Tracking
    1. Convert back to Icy: ImageJ>Tools>Convert to Icy.

2. A scale bar is automatically overlaid onto the stack upon conversion to Icy, if the scale bar plugin is installed [Search using Plugins>Setup>Online plugin]. If needed, edit the scale bar via Inspector window (right side of screen)>Layer tab>Name>Scale bar.
3. Deselect/inactivate the 'eye' icon for Scale bar from the Layer tab>Name to remove the scale bar from the original stack. It can be reactivated on the final stack.
4. Save the newly processed stack by taking a screenshot using the "camera" icon from the Image Window's menu bar, "Take a screenshot of current view" and File>Save as>Tiff….
5. Determine spot sensitivity, if spot sensitivity parameters have already been determined move onto step 6.2.7.
6. Detect spots: select the window with the image or stack to be analyzed, Detection&Tracking>Detection>Spot Detector, and fill in the Settings parameters:
    1. For Input, select "currentSequenceInputDetection" (default).
    2. For Pre Processing, select "Channel 0" (default), or desired channel by cross-referencing the number in the Inspector window>Sequence tab.
    3. For Detector, select "Detect bright spot over dark background;" use "Force use of 2D Wavelets for 3D" only if there are not enough Z-slices in the stacks to perform the analysis. Select "Scale(s)" and "Sensitivity" for each scale (add more scales for larger spots). The Scale and Sensitivity (the larger the number the more sensitive is the detection, a maximum of 140 is suggested by Icy) are trial and error variables, that must be visually checked afterwards and decided upon.
    4. For Region of Interest, use "ROIfromSequence" (default).
    5. For Filtering, use "NoFiltering" (default), or select "SizeFiltering" to define the "Range of accepted objects (in pixels)".
    6. Output: select XLS or XML output setting (select XML format when using 2007 MS Excel or earlier and there are > 65,000 spots). If the spot detector results are used for the tracking analysis, also select "Export to SwimmingPool".
    7. Repeat detection of spots using various scale/sensitivity values until all or most of the spots are detected. Record all of the final parameters.
    8. For colocalization analysis, repeat spot detection for the other channel.

7. To track spots, select Detection&Tracking>Tracking>Spot Tracking>Run the Spot Detector with parameters from step 6.2.6., or use "Select detection results here" pull-down menu to select an existing dataset (for this, keep Spot Detector window open from step 6.2.5). Press the "Estimate parameters" button and select the desired target motion in the Parameters estimation pop-up window (*e.g.* "is both diffusive and directed"). Press the "Run tracking" button.
8. Repeat spot detection and tracking for other channels when tracking spots of multichannel stacks, following steps 6.2.6 and 6.2.7, beginning with the stack generated from step 6.2.7.
9. To visualize tracks, select Detection&Tracking>Tracking>Track Manager – this window opens automatically upon completion of a tracking run. For "Color Track Processor," select "Enable" and choose the desired representation of color for the tracks. Relevant track processors can be accessed via the "add Track Processor…" pull-down menu (*e.g.* select "Track Processor Time Clip," enable the "Track Clipper" window, and choose the desired number of detections to be displayed before and after the current time point.)
10. Save tracks information as an XML track file: Detection&Tracking>Tracking>Track Manager>File>Save as….
11. Save results by taking a screenshot using the "camera" icon from the Image Window's menu bar, "Take a screenshot of current view". Screenshots can be taken with the detected spots and/or the tracks simply by activating/deactivating the corresponding eye icon(s) found in Inspector window>Layer tab>Name>Overlay wrapper.
12. Install the TimeStamp Overlay plugin: Plugins>Setup>Online plugin>TimeStamp Overlay>Install.
13. Add timestamp: Plugins>TimeStamp Overlay (New). Follow instructions on the pop-up window (lower right corner of screen) for directions on placing and formatting the time stamp. The time interval can be added/changed in the Inspector window>Sequence tab>Sequence Properties>Edit.
14. Save results by taking another screenshot. Save image as 1) Tiff format, and 2) as AVI format; for AVI format first convert to RGB rendering (Image/Sequence>Rendering>RGB image).
15. Rotate image to desired orientation: Inspector window>Sequence tab>Canvas>Rotation.
16. Save rotated image by "Take a screenshot of the current view". Ensure the "eye" icon for the scale bar is deselected, as it will also rotate with the image.
17. Choose and crop ROI: select Region Of Interest>2D ROI>Choose ROI shape and then create/draw ROI on the image; Image/Sequence>Plane (XY)>Fast crop.

3. Colocalization analysis
    1. Prepare a colocalization protocol; several examples are provided on the Icy website (http://icy.bioimageanalysis.org/protocol/List) (see **Supplemental Materials**).
    2. Load colocalization protocol: Tools>Scripting>Protocols>Load, and adjust parameters in the interacting blocks (*e.g.* in the "Wavelet Spot Detecting" block use parameters determined in step 6.2.6.).
    3. Measure the size of a particle in pixels, determine the colocalization distance and input it into "Colocalizer" block as "Max distance." Note: The size of the particle in pixels depends on the detection system. To measure size, zoom into a single particle and manually count the pixels that span the width of the signal across. Average the measurements from at least three particles. The maximum distance to be set for colocalization is the size of the particle in pixels (this represents the maximum sum of the radius of two particles touching).
    4. If desired, select one or more ROIs for colocalization analysis: Region Of Interest>2D ROI>Choose ROI shape>Draw ROI on image.
    5. Crop ROI(s): Image/Sequence>Plane (XY)>Fast crop.
    6. Perform colocalization: Protocols editor window>Chosen protocol tab>Run. The final block in Protocols editor window will contain overall colocalization percentage based on spot detection, while information at each time point can be found in the Inspector window>Output tab.
    7. Track colocalized and single particles by following step 6.2.7 (Track spots).
    8. Save as described in step 6.2.16.

Representative Results

Using *PinMol*, several MBs can be designed for one mRNA target (**Figure 1B-C**). After synthesis and purification, the selected MBs are characterized and compared using *in vitro* analysis.
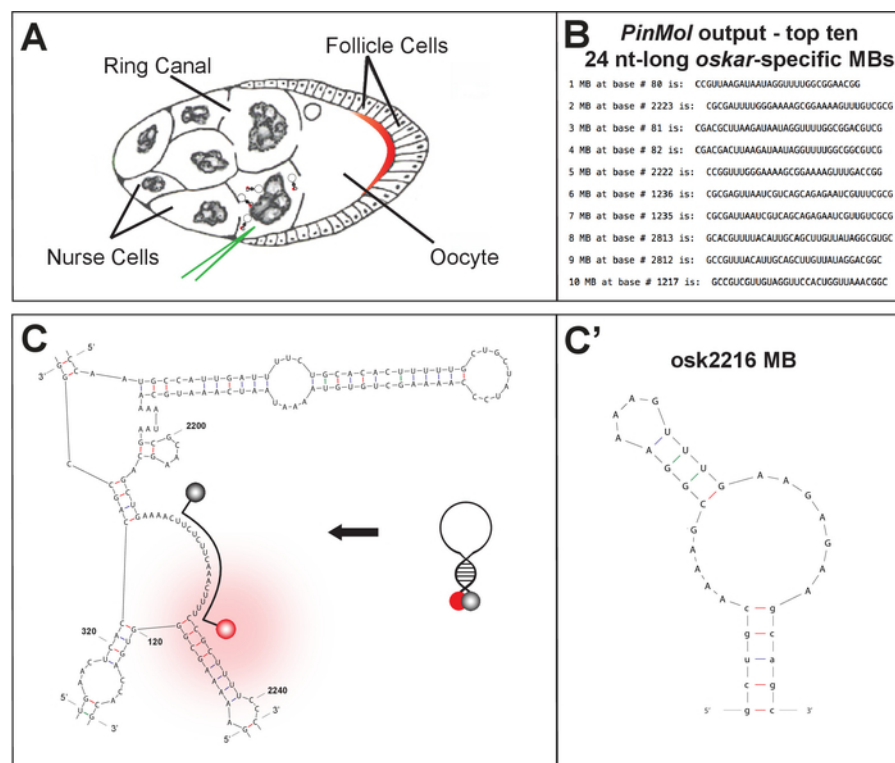

Figure 1: Technique and tissue description for live cell imaging of endogenous mRNAs. (A) Depiction of a mid-stage *Drosophila* egg chamber used for microinjection. The microinjection needle (green) delivers a cocktail of molecular beacons specific for *oskar* mRNA. A quick injection into a nurse cell enables detection of mRNAs in transit to the oocyte, as well as visualization of already localized mRNA at the posterior cortex. (B) *PinMol* software output of molecular beacon ranking for targeting *oskar* mRNA (C) Secondary structure region within *oskar* mRNA targeted by a molecular beacon. (C') Sequence and folding of *oskar*-specific molecular beacon, osk2216. Please click here to view a larger version of this figure.

After optimum performance of MBs is confirmed via *in vitro* characterization, the probes are used for live visualization of endogenous target mRNA(s). It is possible to visualize the patterns of *oskar* mRNA transport and localization at various stages of oogenesis, and in particular at and after mid-oogenesis (7-10) (**Figure 2A-B**). Due to their small size, it is difficult to inject egg chambers at very early stages (1-4). When individually injected in the same stage egg chambers, *oskar*-specific MBs present the same patterns of localization (**Figure 2**, osk1236 vs osk2216).
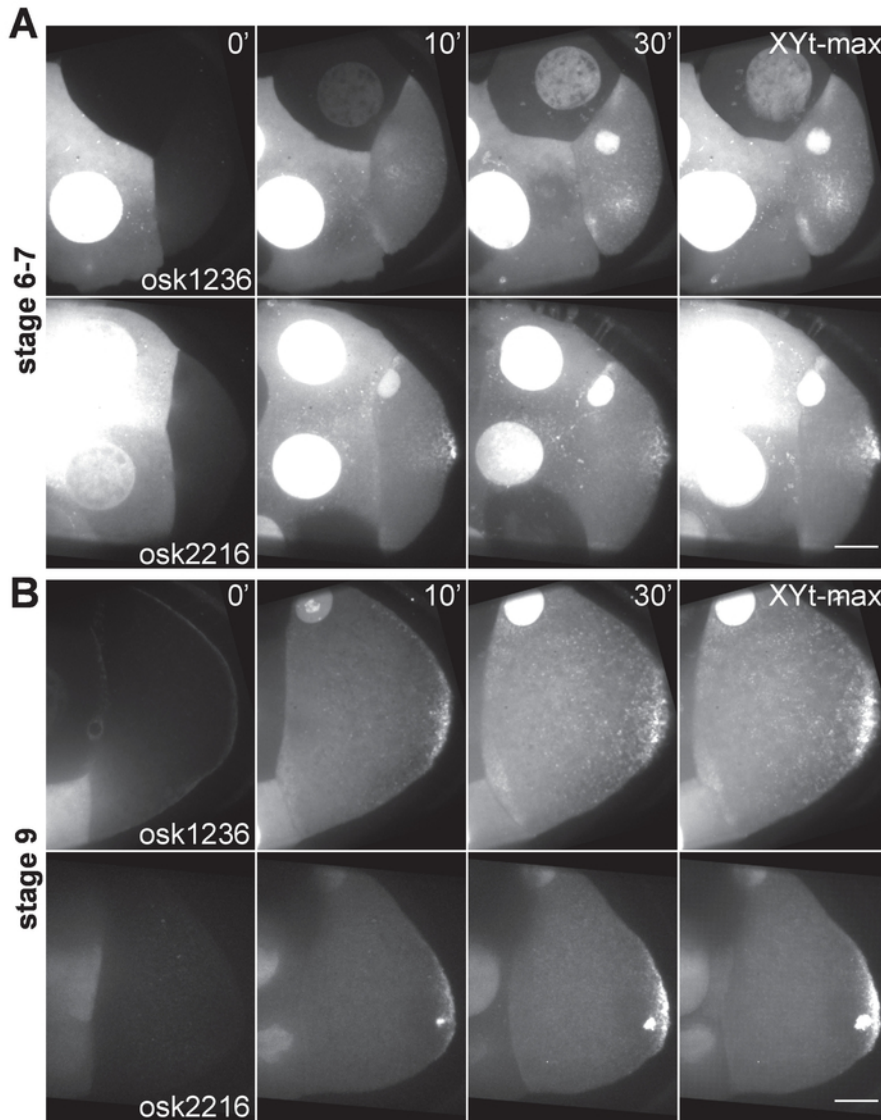
Figure 2: Time sequence of *oskar* mRNA in wild type egg chamber at t = 0, 10, and 30 min time points, after initiation of acquisition. Nurse cell injections of two *oskar*-specific molecular beacons (osk1236 and osk2216) in (A) stage 6-7 egg chambers and (B) stage 9 egg chambers. Scale bar, 20 µm. Please click here to view a larger version of this figure.

Different mRNA targets can be co-visualized by using spectrally distinct fluorescently labeled MBs (**Figure 3**). The MB solution can be microinjected into a nurse cell (**Figure 3A**) or into the oocyte (**Figure 3B**). MBs injected into a nurse cell's cytoplasm will freely diffuse into the other nurse cells as well as into the oocyte, and thus are able to find their target and generate fluorescence signal at other sites than the microinjection site. For example, when performing microinjections in a nurse cell of a late oogenesis stage (9-10) egg chamber, most of the fluorescence signal visualized within the oocyte is generated by the already localized *oskar* mRNA, and less by actively transported transcripts, which are more prevalent in earlier stages (7-8). Note that classical MBs will give rise to non-specific signal within the nuclei, therefore limiting the analysis to the cytoplasmic regions of the egg chamber. Additional modifications, such as NeutrAvidin or gold nanoparticles, have been employed to reduce or eliminate this non-specific signal[28,29]. In spite of this nuclear non-specific signal, the specificity of MBs for *in vivo* detection of *oskar* mRNA has been established using a FRET approach[8], and MB co-injection with *in vitro* transcribed *oskar* mRNA labeled with a fluorophore spectrally distinct from the MB's label[23].
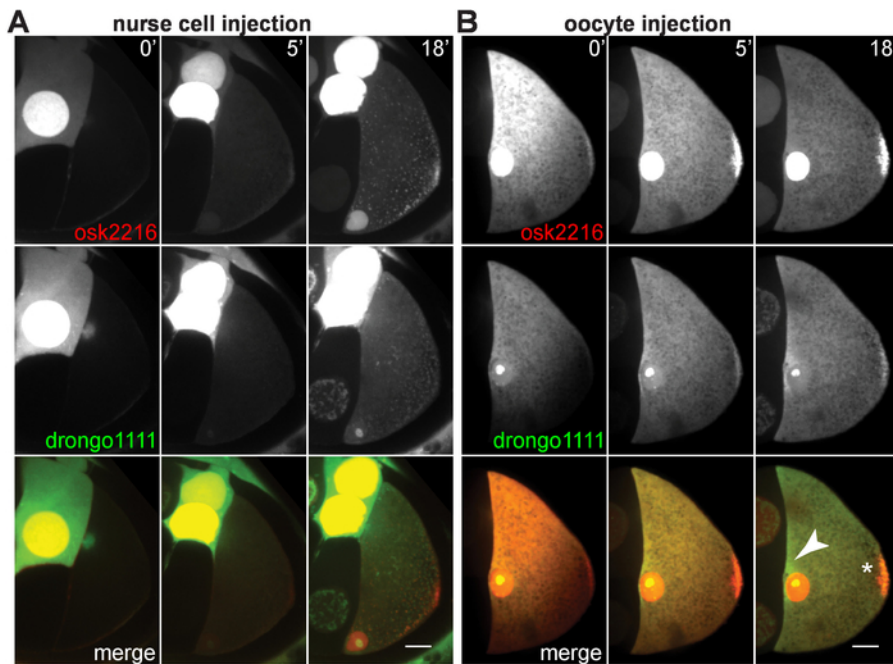
Figure 3: Co-visualization of two mRNA species in live egg chambers. Co-injections of *oskar*- and *drongo*-specific molecular beacons in the (A) nurse cell and (B) oocyte of stage 8-9 egg chambers. *oskar* (red) and *drongo* (green) mRNAs colocalize at the posterior end of the oocyte (asterix), and *drongo* also shows dorso-anterior accumulation (arrowhead). Scale bar, 20 μm. Please click here to view a larger version of this figure.

For mRNA targets that show low expression levels, the fluorescence signal per mRNA molecule is increased by injecting a cocktail solution containing at least two MBs, each binding to different target regions (**Figures 4 and 5**).
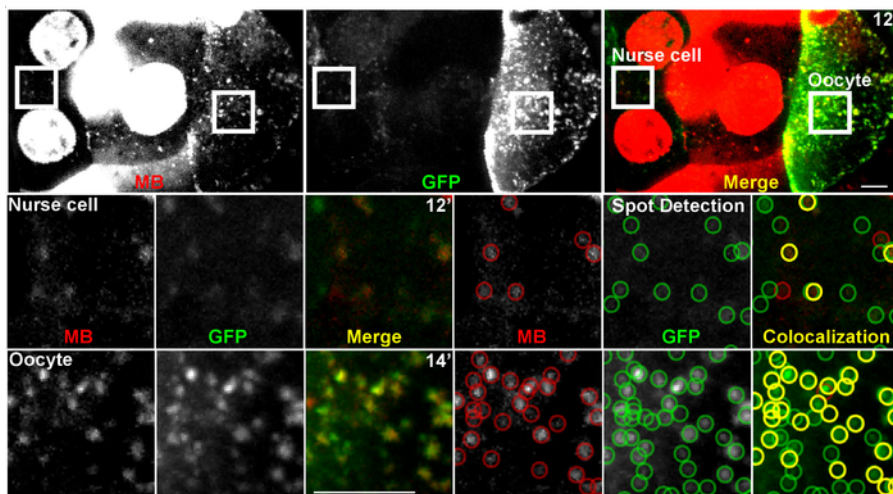

Figure 4: Visualization of *oskar* mRNA with both MBs and MS2-GFP system. Microinjections of a MB cocktail solution (MB) in an egg chamber expressing *osk-MS2::MCP-GFP*[30] (GFP). After the microinjection of a nurse cell, images were acquired every 30 s for 20 min. Two regions of interest were selected, one ROI in a nurse cell and one in the oocyte. Different sensitivity was used for each ROI to detect the spots. ROI nurse cell: Scale 2, Sensitivity 100 for GFP and MB. ROI oocyte: Scale 2, Sensitivity 50 for GFP and 110 for MB. Colocalization distance is 4 pixels for both ROIs. The whole egg chamber and the zoom in on the nurse cell are shown at the 12-min time point, and the zoom in on the oocyte is shown at the 14-min time point. MB spots (red circles) and GFP spots (green circles) identify *oskar* mRNA particles detected by each approach, and the colocalized particles (yellow) indicate where MB and GFP spots are at most 4 pixels apart. XY-projections of 14 Z optical slices at 0.3 μm steps. Acquired as 16-bit data with an 63X objective (oil, NA = 1.4), XY = 0.24 μm, exposure time 500 ms, at 5.23 and 5.39 mW laser power for the 641 nm and 491 nm laser, respectively. Scale bar, 10 μm. Please click here to view a larger version of this figure.
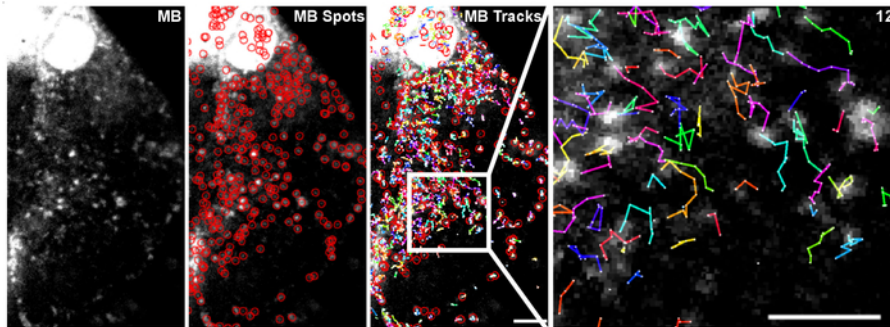
Figure 5: Tracking analysis in the oocyte, after nurse cell microinjection with *oskar* mRNA-specific MBs. MB particles were detected at Scale 2 with Sensitivity 110, and 8 time points are shown before/after the current time frame. MB spots (red circles) are tracked in the volume of the oocyte; tracks represent detection information from 8 time points before/after the shown 12-min time point. Each color represents an individual track. XY-projections of 14 Z optical slices at 0.3 μm steps. Scale bar, 10 μm. Please click here to view a larger version of this figure.

When comparing trafficking of *oskar* mRNA as detected with MBs vs MS2/MCP, the fluorescence signal generated by *oskar*-specific MBs faithfully documents on the transport and localization of transgenic *oskar* mRNA labeled with 10 GFP molecules via the MS2/MCP system (**Figure 4**). In *oskar-MS2/MCP-GFP* transgenic egg chambers at mid oogenesis, acquisition data analysis showed extensive colocalization between fluorescent signals of genetically engineered *oskar-MS2* mRNA detected using MBs and GFP-tagging. At 12 and 14 min post-injection, 57% (7 MB-objects and 13 GFP-objects, with 4 colocalized objects) and 93% (30 MB-objects and 51 GFP-objects, with 28 colocalized objects) of detected MB particles colocalized with GFP particles in the nurse cells and oocyte, respectively. Our analysis yields 31% and 55% colocalization percentages of *oskar-MS2* mRNA with *oskar* mRNA detected with MBs within the cytoplasm of a nurse cell and the oocyte, respectively. Moreover, 5D-stacks can be further analyzed to determine *oskar* mRNA trajectories for long-distance transport in both the nurse cell and oocyte cytoplasm (**Figure 5**).

Discussion

Live visualization of endogenous mRNA trafficking in *Drosophila* egg chambers relies on the use of specific, efficient, and nuclease-resistant MBs, which can now be easily designed with *PinMol* software. MBs are specific probes designed to detect unique sequences within a target mRNA (preferably regions free of secondary structure), making possible highly resolved detection of a transcript. The only limitation when adopting this technique/protocol for other tissues/cell types is the efficiency of MB delivery for the specimen of interest. While other approaches require genetic manipulation of the tissue to express an aptamer and an RNA-binding protein tagged with a fluorescent protein to visualize one target mRNA (*e.g.* MS2/MCP system), multiplexing is possible for, at most, two transcripts. MB technology stands alone for detecting endogenous mRNAs in living cells, and it is the only technique permitting the co-visualization of more than two mRNA species.

MBs can be labeled with a wide range of fluorescent moieties and are stable within the cellular environment when synthesized from modified nucleotides such as 2'-O-methylribonucleotides or locked-nucleic acids[26,31]. These backbone modifications also increase MBs' affinity for their target. Several MBs can be easily designed for target mRNAs of average length. However, some limitations may be encountered for short and/ or highly structured targets. This can be overcome by adopting our tiny molecular beacons, for which the probe region is approximately half of the length of a classical MB probe[31]. Depending on the specimen type, MBs are delivered into cells via electroporation, linking to cell-penetrating peptides, lipofection, or microinjection[23,32,33,34]. An MB's performance efficiency in live cell imaging experiments leans on the capability of the probe sequence to hybridize to the corresponding complementary sequence within the mRNA target, which is determined by the target structure. The predicted MFE RNA secondary structure obtained using *in vitro* measured thermodynamics parameters is valuable in assessing target accessibility, but ultimately it is the *in vivo* target structure and the target interaction with other cellular factors that will determine the MB's suitability for live cell imaging. Genome-wide analysis of RNA secondary structure suggests that many RNAs are less structured *in vivo* than *in vitro*[35]. Although the efficiency of *in vivo* target detection using MBs is mainly dependent on the accessibility of the binding site, optimizing certain MB features will ensure an enhanced visualization of the mRNA target. Specifically, a careful selection of the following parameters should be performed: 1) the probe length can vary between 18 and 26, such that the probe's nucleotide composition is between 31 and 55% GC pairs in the target:MB hybrid, 2) the 5 bp stem sequence should be G/C rich, to maintain the hairpin shape in absence of mRNA target and to provide mismatch discrimination, 3) a modified backbone should be used for protection against nucleases of both MB and target:MB hybrid, 4) the fluorophore/quencher pair can offer an additional modest stability to the MB's stem, and 5) the fluorophore should be stable during long imaging time intervals. In addition, classical MBs usually generate a nuclear non-specific signal[34], which in our case only moderately impacts data processing and analysis. However, for mRNA trafficking visualization at cellular level, this non-specific signal may become problematic. Several groups have proposed modifications or tags, such as tRNA, peptides and nanoparticles, which prevent the delivery of MBs into the nucleus and thus eliminate this possible non-specific signal[33,36].

The biggest drawback of this approach has been the manual design of MBs for live cell imaging. To address this, we have written a Python-based program (*PinMol*) that readily identifies accessible target sites within an mRNA by considering suboptimal secondary structures in addition to the MFE, as well as designs hairpin probes, which are best suited for detection of mRNAs in live cells[24]. *PinMol* uses structural information from secondary structures of the target RNA predicted via energy minimization approaches, and by including information from suboptimal structures, the flexibility or rigidity of specific targeted regions is assessed when designing MBs. It takes into account the accessibility of the targeted regions, as well as the inter- and intramolecular interactions of each selected probe. Additionally, highly regulated stretches of RNA (*e.g.* binding sites for microRNAs or RNA-binding proteins) should not be considered as target sites when selecting probes, as these regions may result in inefficient binding of the MBs. The user can evaluate and eliminate probes targeting these sites, or restrict the target region used by *PinMol* to design probes so it does not include such sites. The relative capability of *PinMol* was demonstrated by comparing the ranking of *PinMol* designed MBs with the experimental results of manually designed MBs[24]. *Pinmol* selected and designed MBs for similar target regions

as well as identified new accessible sites on the mRNA. This is essential for the detection of low copy number transcripts where the fluorescent signal must be increased above background. By scaling up the MB numbers which effectively hybridize to several accessible sites on a target mRNA, signal amplification can be achieved. Therefore, this program facilitates a fast approach to design multiple MBs per target mRNA, and to simultaneously visualize numerous mRNAs in a live cell.

In order to achieve high quality 5D (XYZCt) acquisition data of transported mRNAs within the egg chamber, proper dissection of individual egg chambers and effective microinjection into nurse cells are critical. For studies during early stages of development, microinjection can be detrimental to the viability of the egg chamber and thus the length of a live cell imaging experiment is shortened (< 20 min). An increased success rate of the microinjection experiments can be ensured by using commercially available ultrafine needles. In addition, a quick set-up of the acquisition settings is important so that the early, post-injection time points can be captured. The quality of the spot detection and tracking data will only be as good as the quality of the images acquired.

Upon image acquisition, it is essential that subsequent analysis steps are also completed carefully and precisely. Although post-acquisition processing and analysis offer their own set of difficulties, they can be streamlined by choosing the appropriate software for one's particular experiment or sample. Current existing programs include Volocity (PerkinElmer), Imaris (Bitplane), ImageJ/Fiji and Icy[37]. Of the three, Icy offers several benefits, as it is an open community platform that allows for, both, processing (via ImageJ) and analysis of imaging data. Here we describe the steps necessary for efficient processing and analysis of RNA imaging data using Icy. Our results are representative of data obtained by co-visualizing two mRNA species in a whole egg chamber and by tracking an mRNA via the MB technology and the MS2/MCP system.

The post-acquisition processing with Icy software provides user flexibility in the image processing (*e.g.* brightness, contrast) and analysis (*e.g.* the thresholds/cut-offs settings for sensitivity of detection of the fluorescent particles as spots). Icy also enables the control relevant parameters within each block of the Protocols Editor run (*e.g.* determine the colocalization distance and use it as "Max distance" into the "Colocalizer" block). Icy software is updated at launch, and has reliable online support to troubleshoot any problems. The described protocol was designed for detecting *oskar* mRNA and the representative results were obtained using parameters optimized for the kind of mRNA particle to be detected and tracked. For example, *oskar* mRNA is an abundant transcript that is predominantly transported during mid stages of oogenesis, utilizing the microtubule network and dynamically associating with various protein factors throughout development of the oocyte. We previously reported that *oskar* mRNP undergoes extensive remodeling during transport from the nurse cells into the oocyte[23]. In addition, using MBs we characterized the temporal and spatial characteristics of endogenous *oskar* mRNA trafficking, and found that hundreds of *oskar* transcript copies can be incorporated to form large *oskar* mRNPs.

Post-acquisition analysis for colocalization and tracking can also be performed by using other software such as Imaris, ImageJ/Fiji and Volocity. Icy was selected for its capability to threshold and annotate fluorescent particles with high sensitivity and tracking capabilities. Here, we describe object-based colocalization, but colocalization, albeit without tracking, can also be quantified by determining the overlap and degree of colocalization using PCC(Costes) analysis using Icy and ImageJ plugins (Colocalization studio, JACoP)[38,39].

In the future, an optimized, long-term imaging protocol is desired to ensure extended egg chamber survival. This would provide longer acquisition in order to analyze data pertaining to long-range mRNA trafficking studies.

Disclosures

The authors have no conflict of interest to disclose.

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