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Visualizing and Tracking Endogenous mRNAs in Live Drosophila melanogaster Egg Chambers

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May 31, 2018

Dear Dr. Mukherjee,

Thank you for the invitation to submit a JoVE manuscript.

We are pleased to submit an article entitled “Visualizing and Tracking Endogenous mRNAs in Live *Drosophila melanogaster* Egg Chambers”, where we describe a comprehensive protocol for designing and delivering molecular beacons into *Drosophila melanogaster* egg chambers as well as performing data analysis to track endogenous mRNAs in their native cellular environment.

Best wishes,

Irina E. Catrina, PhD
Diana P. Bratu, PhD

TITLE:

Visualizing and Tracking Endogenous mRNAs in Live *Drosophila melanogaster* Egg Chambers

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KEYWORDS:
Molecular beacon, live cell imaging, endogenous mRNA visualization, mRNA trafficking, *Drosophila melanogaster* egg chamber, egg chamber microinjection, colocalization, particle tracking.

SUMMARY:
Here, we present a protocol for the visualization, detection, analysis and tracking of endogenous mRNA trafficking in live *Drosophila melanogaster* egg chamber using molecular beacons, spinning disc confocal microscopy, and open-source analysis software.

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[1-3]. 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)[10-13], 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**

1.1. 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).

1.1.1. 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.

1.1.2. 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.

1.2. 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/).

1.2.1. 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).

1.2.2. 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.

1.3. 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

2.1. 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].

2.2. 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].

2.3. 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.

2.4. 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.

2.5. 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**

3.1. Feed newly hatched, mated females for 2-3 days with fresh yeast paste.

3.2. 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.

3.3. 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.

3.4. 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

4.1. 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.

4.2. Select the objective. A 40X oil objective is recommended for finding an appropriate egg chamber and for performing microinjection.

4.3. 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 A→P axis perpendicular to the needle tip to allow for easy injection within a nurse cell proximal to the oocyte).

4.4. 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.

4.5. Set-up the injector with injection pressure of 500-1,000 hPa and compensation pressure of 100-250 hPa (see **Table of Materials**).

4.6. Slowly move the stage to bring in the field of view an area of the oil drop void of egg chambers.

4.7. 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.

4.8. Perform a 'clean' function to remove the air from the tip of needle and to ensure that there is flow from the needle.

4.9. 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.

4.10. Perform a fine adjustment of the objective's Z-position such that the membrane separating the follicle cells from nurse cells is in focus.

4.11. Insert the needle into a nurse cell and perform injection for 2-5 s.

4.12. Gently remove the needle and retract it to the home position.

4.13. 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.

5.1. Set up acquisition protocol to record an XYZCt stack of 8-16-bit images (XYZ = volume, C = channel, t = time).

5.2. 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.

5.3. Select the Z step (*e.g.* 0.3 µm), and the top and bottom Z limits (*e.g.* -2 µm to 2 µm).

5.4. Input the acquisition time and sampling rate (*e.g.* every 15-30 s for up to 1 h).

5.5. Initiate acquisition.

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

6.1. Image Processing

6.1.1. Download, unpack, and open Icy, an open community platform for bioimage informatics (http://icy.bioimageanalysis.org/)

6.1.2. Open the XYZCt stack acquired in step 5: Image/Sequence >File>Open.

6.1.3. Convert stack to ImageJ: ImageJ> Tools> Convert to IJ, have Detached Mode ON.

6.1.4. 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.

6.1.5. Save substack as TIFF file: ImageJ>File>Save As>Tiff…; use this file for subsequent steps.

6.1.6. Split channels: ImageJ>Image>Color>Split Channels.

6.1.7. 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 flurorophore. 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.

6.1.8. Adjust the brightness and contrast for each channel: ImageJ>Image>Adjust>Brightness/Contrast, select Apply.

6.1.9. Save each channel as a separate TIFF file: ImageJ>File>Save As>Tiff....

6.1.10. 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).

6.2. Spot Detection and Tracking

6.2.1. Convert back to Icy: ImageJ>Tools>Convert to Icy.

6.2.2. 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.

6.2.3. 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.

6.2.4. 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….

6.2.5. Determine spot sensitivity, if spot sensitivity parameters have already been determined move onto step 6.2.7.

6.2.6. Detect spots: select the window with the image or stack to be analyzed, Detection&Tracking>Detection>Spot Detector, and fill in the Settings parameters:

6.2.6.1. For Input, select "currentSequenceInputDetection" (default).

6.2.6.2. For Pre Processing, select "Channel 0" (default), or desired channel by cross-referencing the number in the Inspector window>Sequence tab.

6.2.6.3. 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.

6.2.6.4. For Region of Interest, use "ROIfromSequence" (default).

6.2.6.5. For Filtering, use "NoFiltering" (default), or select "SizeFiltering" to define the "Range of accepted objects (in pixels)".

6.2.6.6. 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".

6.2.6.7. Repeat detection of spots using various scale/sensitivity values until all or most of the

spots are detected. Record all of the final parameters.

6.2.6.8. For colocalization analysis, repeat spot detection for the other channel.

6.2.7. 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.

6.2.8. 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.

6.2.9. 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.)

6.2.10. Save tracks information as an XML track file: Detection&Tracking>Tracking>Track Manager>File>Save as….

6.2.11. 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.

6.2.12. Install the TimeStamp Overlay plugin: Plugins>Setup>Online plugin>TimeStamp Overlay>Install.

6.2.13. 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.

6.2.14. 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).

6.2.15. Rotate image to desired orientation: Inspector window>Sequence tab>Canvas>Rotation.

6.2.16. 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.

6.2.17. 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.

6.3. Colocalization analysis

6.3.1. Prepare a colocalization protocol; several examples are provided on the Icy website (http://icy.bioimageanalysis.org/protocol/List) (see **Supplemental Materials**).

6.3.2. 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.).

6.3.3. 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).

6.3.4. If desired, select one or more ROIs for colocalization analysis: Region Of Interest>2D ROI>Choose ROI shape>Draw ROI on image.

6.3.5. Crop ROI(s): Image/Sequence>Plane (XY)>Fast crop.

6.3.6. 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.

6.3.7. Track colocalized and single particles by following step 6.2.7 (Track spots).

6.3.8. 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.

[Insert **Figure 1** here]

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).

[Insert **Figure 2** here]

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].

[Insert **Figure 3** here]

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**).

[Insert **Figure** 4 here]

[Insert **Figure 5** here]

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**).

FIGURE AND TABLE LEGENDS:

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.

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.

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.

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.

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.

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[23,32-34]. 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.

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DISCLOSURES:

The authors have no conflict of interest to disclose.

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Figure 1

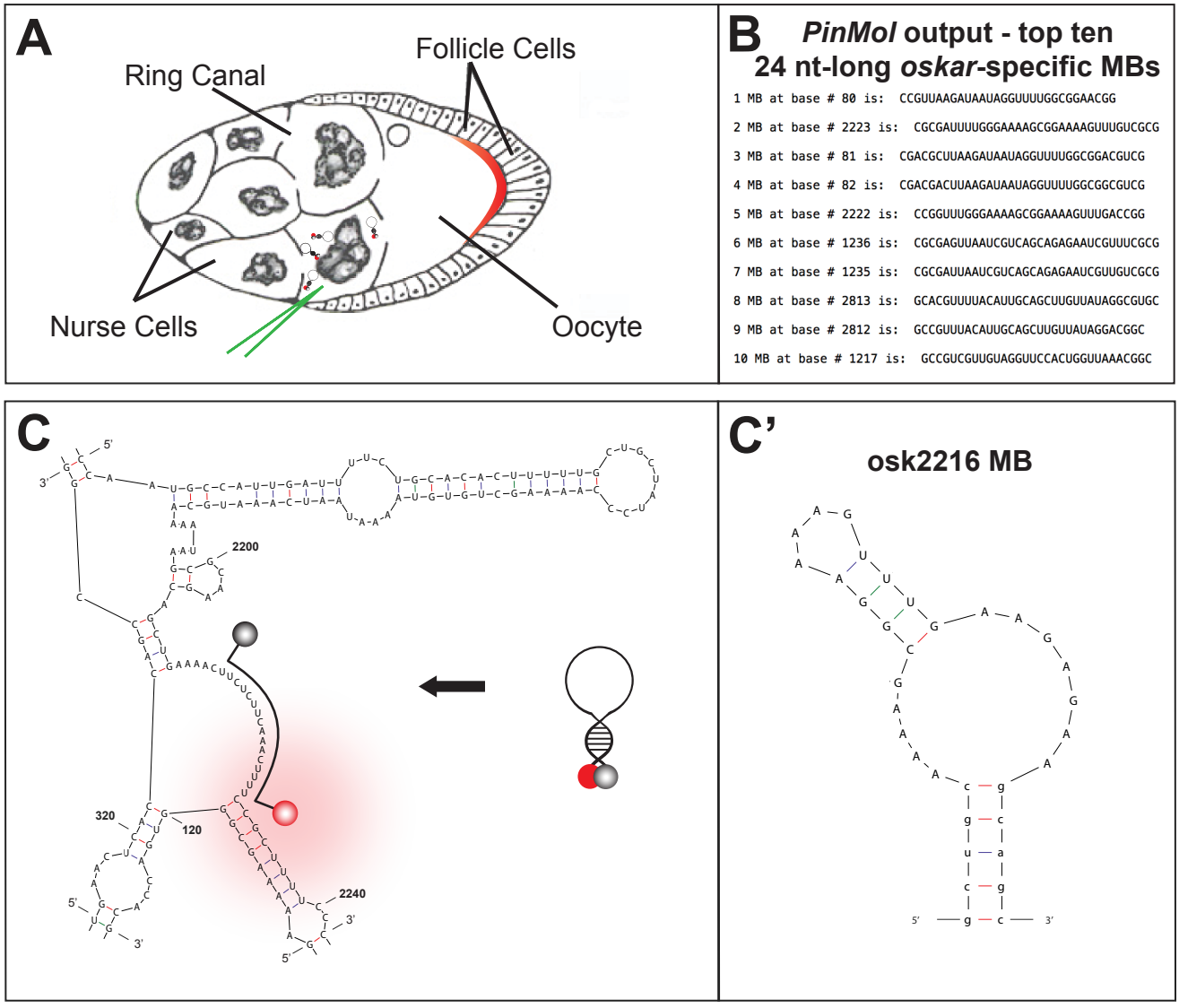

Figure 2

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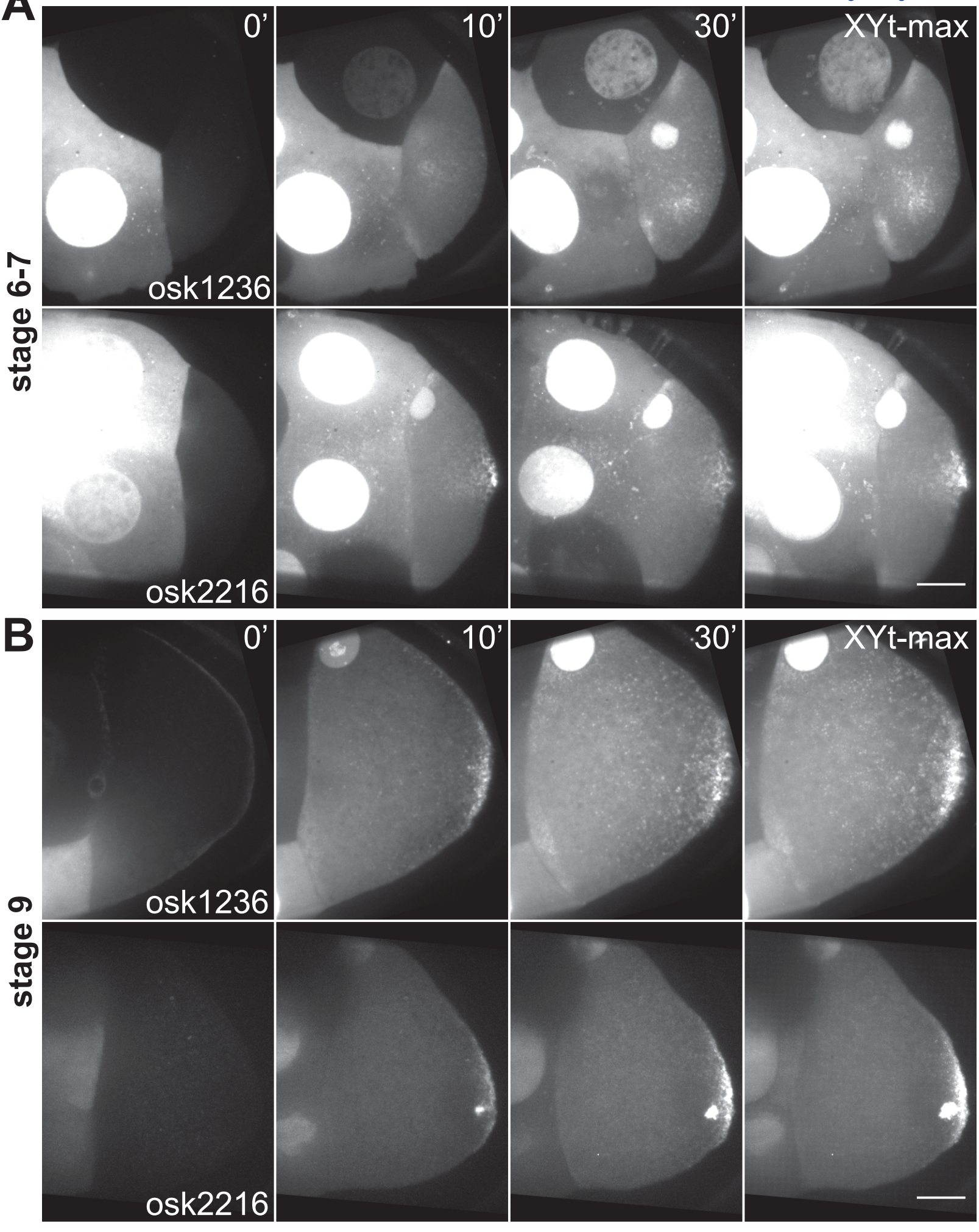

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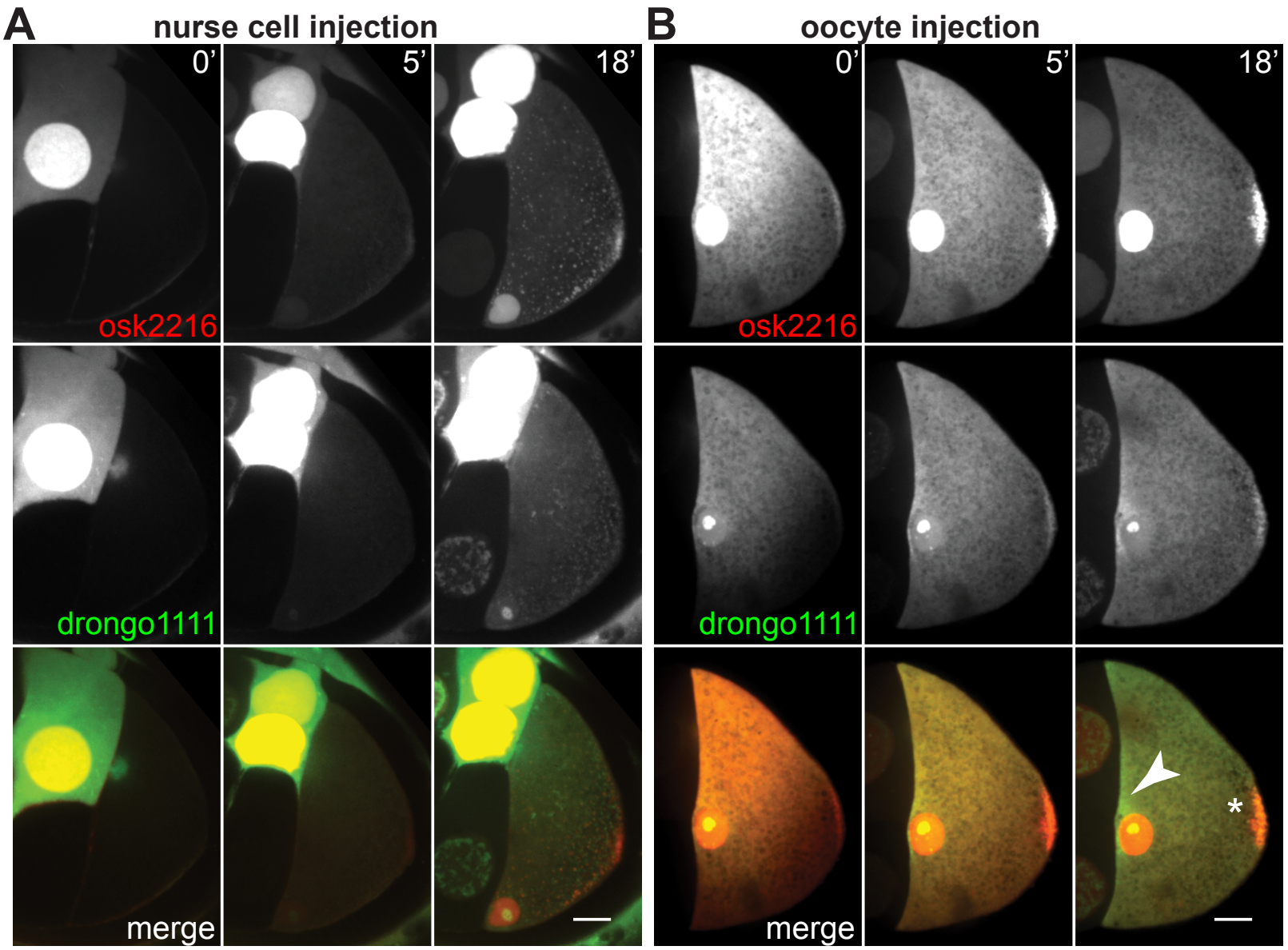

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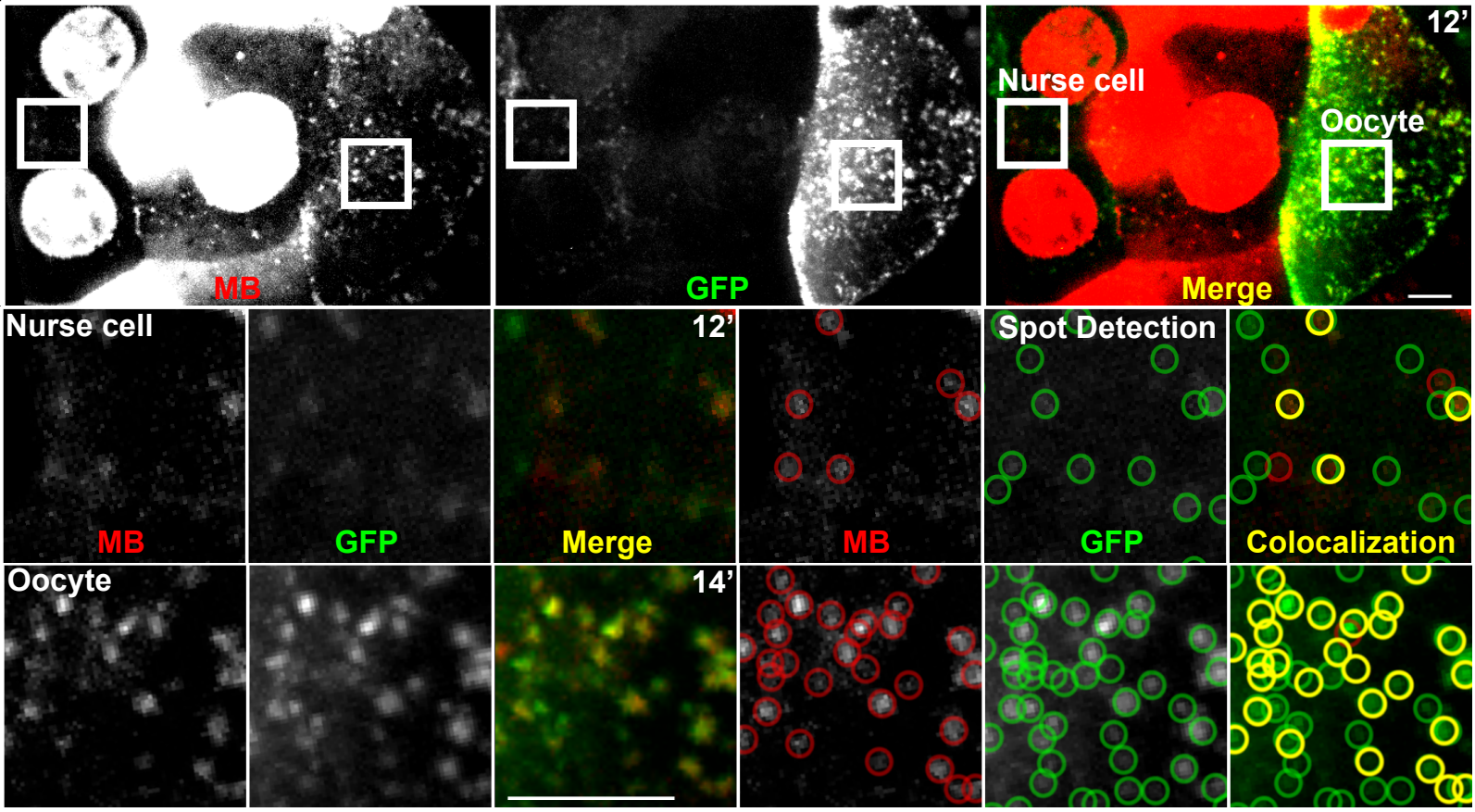

Figure 5

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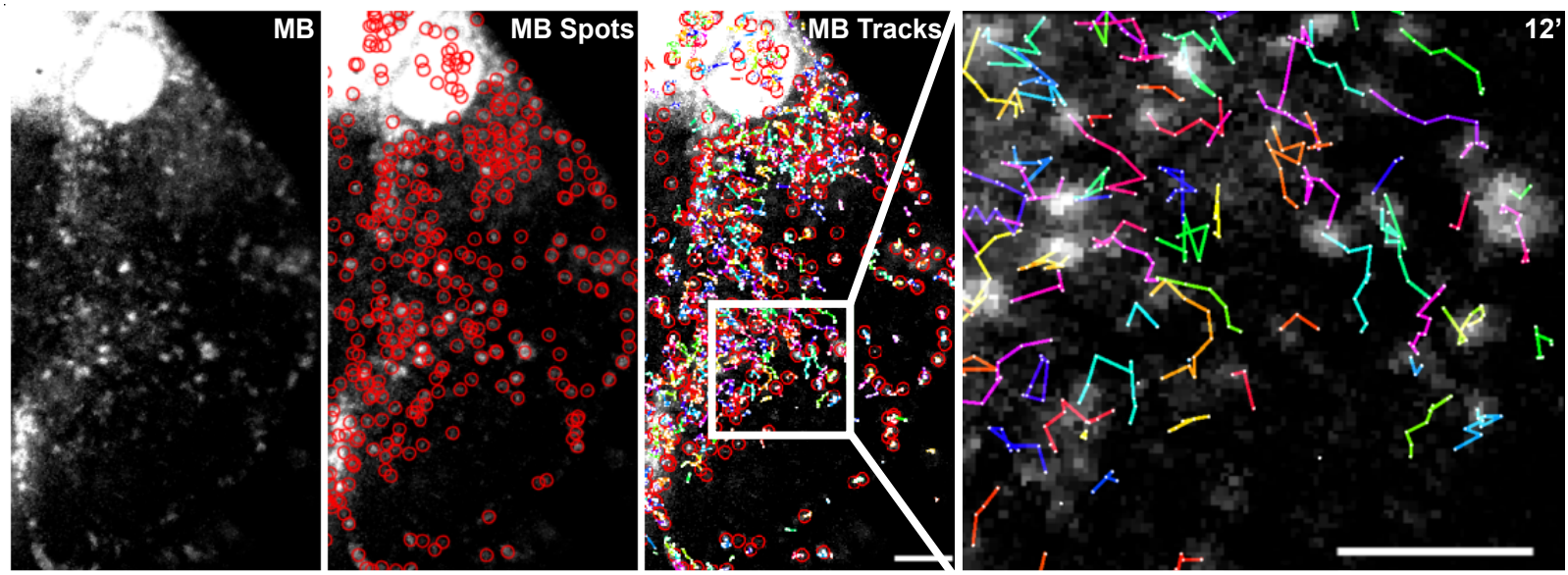

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Spectrofluorometer	Fluoromax-4 Horiba-Jobin Yvon	n/a
Quartz cuvette	Fireflysci (former Precision Cells Inc.)	701MFL
Dumont #5 tweezer	World Precision Instruments	501985
Halocarbon oil 700	Sigma-Aldrich	H8898
Cover slip No.1 22 x 40mm	VWR	48393-048
Dissecting microscope	Leica MZ6 Leica Microsystems Inc.	n/a
CO2 fruit fly anesthesia pad	Genesee Scienific	59-114
Tris-HCL pH7.5	Sigma-Aldrich	1185-53-1
Magnesium chloride	Sigma-Aldrich	7791-18-6
NaCl	Sigma-Aldrich	7647-14-5
Spinning disc confocal microscope	Leica DMI-4000B inverted microscope equipped with Yokogawa CSU 10 spinning disc Leica Microsystems Inc.	n/a
Hamamatsu C9100-13 ImagEM EMCCD camera	Hamamatsu	n/a
PatchMan NP 2 Micromanipulator	Eppendorf Inc.	920000037
FemtoJet Microinjector	Eppendorf Inc.	920010504
Injection needle: Femtotips II	Eppendorf Inc.	930000043
Loading tip: 20ul Microloader	Eppendorf Inc.	930001007
Micro Cover glasses no. 1 or 1.5, 22x40mm	VWR	48393-026; 48393-172
Dry yeast	Any grocery store	n/a
Computer, > 20 GB RAM		

Comments/Description
Photon counting spectrofluorometer
Thin tweezers are very important to separate out the individual egg chambers
Although processing can be carried out on most computers, higher capabilities will increase the speed of the processing

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Rebuttal Document for JoVE58545 manuscript

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We performed further proofreading of the manuscript.

2. Please revise lines 98-100 to avoid previously published text.

We revised these lines, and are now included in the DISCUSSION section:

"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."

3. Figure 1: There is no panel A' but panel C' in Figure 1. Please describe panel C' in the figure legend.

The figure legend was corrected and re-arranged to match Fig. 1.

4. Figure 3: Please define the arrowhead and asterisk symbol in the figure legend.

The missing info was added in the Fig. 3 legend.

5. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

The short abstract was rephrased, as requested:

"Here, we present a protocol for the visualization, detection, analysis and tracking of endogenous mRNA trafficking in live *Drosophila melanogaster* egg chamber using molecular beacons, spinning disc confocal microscopy and open-source analysis software."

6. The current Long Abstract is over the 150-300 word limit. Please shorten it.

The long abstract was shortened, and now contains 292 words.

7. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Spaces were added before all measurement units.

8. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

The protocol was revised as suggested.

9. Please discuss Fig. 1B in the manuscript.

Fig. 1B is now referenced in the text.

10. Discussion: Please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique.

This information was added in the Discussion section and is also described in our response to the reviewers' critiques/requests.

11. References: Please do not abbreviate journal titles.

We edited the final document after references were converted to text.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors present a potentially highly valuable method for labeling endogenous mRNAs in living Drosophila oocytes using molecular beacons. A method like this has the potential to complement and extend the current bacteriophage stem loop-mediated labeling of artificial reporter transcripts, but this method has not been widely adopted. The protocol presented here could provide a valuable resource.

Major Concerns:
However, in its current form, the manuscript leaves out important details that potential users would find very helpful if they wish to adopt the method. There are two major areas that must be addressed.

1) *First, the criteria for designing well-behaved MBs must be explicitly described.*

We included the following information in the DISCUSSION section, and most of it is extensively covered in the literature of specialty:

"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]."

2) *Second, and perhaps more importantly, the degree of nonspecific labeling appears to be massive, at least in nuclei, vastly outweighing the putatively specific signal, as judging from Figures 2 and 3. Any reader of this protocol will be rightfully skeptical about the reliability of the method, given the data presented. These issues should be addressed before the manuscript is suitable for publication.*

The nuclear non-specific signal generated by MBs is well documented in the literature. In our case our analysis is not impeded by it and therefore we have not employed any of the modification optimized by other groups that minimize this non-specific signal. In addition, MS2/MCP tagging, the main alternative method for visualization of RNA transport in live cells, also generates a non-specific fluorescent signal in the nucleus. We included this information in the DISCUSSION section:

"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]."

Comments follow:

3) *Section 1: Design of MBs: As written, the protocol provides no information regarding the criteria for MB selecting sequences. Overall, much more guidance is needed. It seems particularly important to provide as much information as possible regarding MB design given that the authors state "The biggest drawback of this approach has been the design of MBs for live cell imaging." Could the authors describe something about their experience designing these probes to give a new user some hints about features of well-behaved versus poorly performing probes?*

We now include general guidelines to design MBs in the DISCUSSION section, also see our response for the first major area to be addressed. The specific details on how *PinMol* works to include our recommendations for designing MBs make up a whole new manuscript that is available on *bioRχiv*, as referenced in this manuscript, and which is currently pending review at the *RNA* Journal.

4) *1.1.1 Could the authors briefly describe the use of the RNA folding website. It is not clear what exactly is the goal of this step.*

The goal is mentioned in the main step 1.1, specifically, to obtain the predicted secondary structures for the target mRNA of interest. We re-worded the description of step 1.1.1. (please see below, *Reviewer 1 comment #7*).

5) *Should the user try to maximize the number of probes, or some features of their sequence?*

At this step there are no probes designed, this is only to predict the TARGET secondary structures, which are comprised of the minimum free energy structure (MFE) and a user-dependent number of sub-optimal structures.

6) *What is meant by "5 or 10% sub-optimality"?*

This defines how many suboptimal structures will be considered to generate the file (ss-count file) that is used as input for the *PinMol* analysis. X% sub-optimality means that structures that have a free energy value that is within 5% of the MFE value will be included in the analysis.

7) *What is the effect on the number of potential MBs of choosing either 5 or 10%? Can the user choose other values (0? 20?)? Should values other than 5 or 10 be avoided? What is the upper bound of number of computed foldings?*

This depends on each mRNA and its sequence. We have not analyzed a range of sub-optimality, but we found that using 5 or 10% improves the MB design by identifying regions within the target mRNA that may be flexible or rigid. This is also described in the *PinMol* manuscript.

We modified this step to include more details, and included a new Note, as follows:

"1.1.1. 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 1). "

"Note 1: 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."

8) *1.2.2 Please state explicitly what the user should do after "comparing with any positive BLAST hits."*

The following sentence was added:

"Eliminate probes that show > 50% cross-homology with other mRNAs that are also expressed in the tissue/cell of interest."

9) *1.3 Are there resources where a reader could find a list of fluor-quencher pairs?*

The reference relevant to MB design is now provided.

10) *Note 2: "A more rigorous characterization" Please say explicitly that the more rigorous assay is obtained by using in vitro synthesized RNA instead of DNA oligos in the thermal denaturation assay in section 2.3, if this is what the authors mean to indicate. Otherwise it is not clear what is the more rigorous assay, or what comparison is being made.*

This is now Note 3, and was modified as suggested:

"Note 3: 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]."

11) *2.1 When the authors say to purify by HPLC, one suspects they are referring to purification of MBs from in-house synthesis and not to those obtained by vendors. Please rearrange this section to clarify. For those users wishing to perform HPLC on their in-house synthesized MBs, please provide either more details on HPLC setup or an explicit reference (e.g. reference 7).*

Oligonucleotides prepared using either method should be purified using HPLC. A commercial provider will have a mandatory purification (gel or HPLC) for this type of oligonucleotides. We modified the text as suggested:

"2.1. Use in-house synthesis and purification as previously described[7], or services from commercial providers, to synthesize and purify one to five MBs (see Note 2), 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."

12) *2.3 Please briefly describe the assay of thermal denaturation and what the user should be looking for. One imagines that MBs in vitro might exhibit different behavior from in vivo behavior. In cases where the denaturation assay suggests a probe is poorly behaved, is it ever worthwhile to check its behavior upon injection? Conversely, how often do MBs that perform well in vitro yield no signal or only nonspecific signal in vivo?*

This step was split into three steps, and edited as follows:

"2.3. Perform thermal denaturation of the MB alone and measure its melting temperature (Tm) and confirm that the MB assumes the desired hairpin shape at physiological temperature. We observed Tm values between 60 and 90 °C.

2.4 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.

2.5 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*."

Line 198 has a reference to a section 2.4, but there is no section 2.4.

This is now true after step 2.3 was split.

13) *3.4 Describe why it is necessary to remove the youngest stages. Which stages do the authors consider the youngest?*

This step was edited to include the requested information:

"3.4. 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 (see Note 4)."

14) *Section 4 Perhaps include a reference to the "table of materials" so that a reader can find some information about the injection apparatus.*

We included the requested reference.

15) *Sections 5:*
-a reference to the table of material might be helpful so the reader can see what confocal setup is used.

We included the requested reference.

16) *-Could the authors describe in more detail some of their imaging parameters? For example, what is the physical size represented by the voxels in their image stacks? What is the pixel dwell time? Do the authors have an estimate of the laser power applied to the sample? Some guidelines will be helpful for a potential user to get started with the*

This comment appears to be incomplete. We are providing the requested details for Fig. 4, but these will be different for different microscope set-ups, and depend heavily on the sample and RNA target of interest, quality of the objective, type of camera used, age of the set-up, etc. The details are now provided in the Fig. 4 legend. Instead of the pixel dwell time, which can be varied in a single-point laser scanning setup, we provided exposure times for our acquisition protocol with the spinning disc confocal microscope.

17) *Section 6 processing:*

-The header to section 6 says "using Icy and/or ImageJ, but the protocol switches between the 2 programs. This seems like extra work if the preparation can be done in only Icy, which is what the header to Section 6 implies.

ImageJ is incorporated within Icy, basically only Icy is used, ImageJ is not separately opened.

18) *-Authors should recommend criteria for determining background/nonspecific fluorescence. If MB binding is specific, then one imagines the background will be quite low and the majority of the signal will be "true" signal.*

Within the cytoplasm and in *in vitro* hybridization experiments, the background signal mainly arises from how effective the quenching of the fluorophore is, and not from non-specific binding. Lower performance can be due to the type of fluorophore/quencher pair and an ineffective quencher, weak stem region, improper MB folding. Usually this is indicated with the signal:background (S:B) ratio for each MB, which is determined during the characterization of the MB using *in vitro* hybridization. However, as the reviewer noted, the *in vivo* and *in vitro* performance of the MB will likely differ. As an example we provided the S:B measured using the corresponding DNA target oligonucleotides, for osk1236 and osk2216 in step 6.1.6:

"6.1.6. 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". Background signal will mainly arise from improper quenching of the flurorophore. 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."

19) *-Related to the preceding comment, what is the detection threshold for this method? That is, can MBs detect single mRNA molecules? Given the high density of osk in oocytes and nurse cells, one suspects that single mRNA detection is out of the question. On the other hand, the labeling might be very efficient, so perhaps single molecule detection is possible. Can the authors give any estimate of the minimum number of mRNAs per osk RNP that their MBs can detect?*

As mentioned in the manuscript for Figs. 4 and 5, the detection performance can be improved by preparing several MBs for one mRNA target. And, we previously reported a detailed characterization of *oskar* mRNPs trafficking, where we found that hundreds of mRNA copies can make up large *oskar* mRNPs, which are dynamically remodeled during transport. We included the following information in the DISCUSSION section:

"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."

Line 474 there is a reference to a Fig. 6, but the authors mean Fig. 5.

The text was corrected.

20) *Figures and legends:*

Fig 1: The legend does not correspond to the figure. A and A' refer to panels labeled C and C', B refers to panel labeled A, and C refers to B. It appears osk2216 is not listed in panel B. It would be preferable if the MB shown in panel C' was the same as the one depicted in its binding conformation in C.

We rectified this oversight.

21) *Fig 2: How many nurse cells were injected? Can the authors please comment on the appearance in Fig 2A, top row, of fluorescence in what appears to be an uninjected nurse cell? I assume this*

is due to MB entering this NC from the oocyte and not from "retrograde" osk mRNA transport, but this should be stated explicitly.

We perform microinjection in only one site, which can be the nurse cell cytoplasm or the ooplasm. When injected in the nurse cells the MBs freely diffuse into other nurse cells, which are inter-connected and connected with the oocyte through ring canals (see Fig. 1A), and different nurse cells can have can have different "uptake efficiency" of the MB depending on its relative position to the injection site. In Fig. 2A, for t = 0 min panel only the injected nurse cell presents fluorescence signal, and MB from the injected nurse cells is also present in the other nurse cell in later time points. We included this detail in REPRESENTATIVE RESULTS section:

"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."

22) *The degree of signal from the nurse cell nuclei and the germinal vesicle is massive and very striking. The authors say this arises from "classical MBs" that "give rise to nonspecific signal within nuclei." This massive nonspecific labeling seems to be the main limitation of this method. Why is the signal so strong in nuclei? The authors briefly mention ways to reduce non-specific signal. Given the huge nonspecific signal, shouldn't such modifications be used by default? Why should the reader believe that objects detected in the cytoplasm are in fact the mRNAs of interest? Can the authors please provide evidence, reasoning, or references regarding the degree of nonspecific labeling within the cytoplasm? Without some explicit discussion of the nuclear (and potentially other) non-specific signal, a potential user of this protocol will look upon the method with no small degree of skepticism about its usefulness.*

The specificity of MBs is well established [Bratu, D. P. et al. *Proc Natl Acad Sci U S A.* **100** (23), 13308-13313, (2003); Mhlanga, M. M. *et al. PLoS One.* **4** (7), e6241, (2009)]. We have confirmed the specificity of MBs in detection of the mRNA of interest by co-injecting *oskar*-specific MBs with *in vitro* transcribed *oskar* RNA labeled with a fluorophore spectrally distinct from the one used to label the MB. More importantly, we confirmed the MBs specificity in detecting endogenous *oskar* mRNA using a FRET approach with two MBs binding to adjacent sites on the mRNA target. The non-specific nuclear signal is sequestered in the nucleus is not believed to be exported within the nurse cell cytoplasm. We included this information in the REPRESENTATIVE RESULTS section:

"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]."

23) *In the legend to Figure 3, please describe what features are being highlighted by the asterisk and arrowhead.*

Please see Editorial comment #4.

24) *In Figure 4, what is the physical distance represented by 4 pixels? Is this 4 pixels in XY alone or in Z as well? In the legend there is not much information conveyed by e.g. "scale 2, sensitivity 50." This doesn't help the reader understand the quantities of interest, such as the probability of successfully detecting an osk RNP, or the fraction of signals that are false positives.*

The object-based colocalization protocol performs object detection in each Z, therefore the 4 pixel distance refers to the XY plane, and the pixel size is now provided (see also *Reviewer 1 comment #16*). The scale and sensitivity are determined by trial and error and depend on the data analyzed. Object-based colocalization allows one to visually inspect the data and find any false positives. In our analyses we found that low-intensity objects will not be detected, rather than the background signal to be identified as an object. Icy offers additional colocalization methods, but we chose the object-based colocalization to show the detected objects.

25) *The authors state that 57% and 93% of MB particles are colocalized with an MS2 particle. This implies that this mean that 43% and 7% of MB particles are not MS2-containing particles. The reader will also be interested in the converse, the fraction of MS2 particles that are labeled by MBs. From the images presented, it appears that the majority of MS2 particles are not labeled by MBs. Please provide some measurement of the degree of labeling of MS2-containing particles. Please also provide the number of objects assayed to arrive at these percentages. This will help give the reader a sense of the detection efficiency.*

The relevant text within the REPRESENTATIVE RESULTS was edited to include this information:

"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."

26) *Moreover, some explanation is needed for the percentages that are presented. At first glance, if the MS2-labeled transcript is expressed from a transgene in a genetic background with normal levels of endogenous osk expression, then there will be at least as many (and likely more) non-MS2-containing osk transcripts as MS2-containing ones. So one naively expects a large fraction, and likely the majority, of MB particles not to colocalize with MS2, whereas the authors report the opposite. (Please provide the genotype and a reference for the osk-MS2 and MCP-GFP transgenic lines).*

Premature and overexpression and/or ectopic expression of Oskar protein is toxic to egg chamber development and is embryonic lethal, therefore the *oskar*-MS2 transgene was generated in an *oskar*-

null background. The *oskar*-MS2/MCP-GFP construct consisted of 10 MS2-binding sites inserted into an Spe1 site that was introduced immediately after the *osk*ar stop codon in a osk^{A87}/Df(3R)pXT103 background [Zimyanin, V.L., et al. *Cell* **134** (5), 843-853, (2009)]. The reviewer's statement and inquiries assume that all transgenic *oskar* mRNA transcripts are labeled with MCP-GFP. The *oskar*-MS2 system only claims that the GFP cytoplasmic signal (and not the non-specific nuclear GFP signal) is representative of endogenous *oskar* mRNA trafficking. To our knowledge it has not be shown that the *oskar*-MS2 transgene only expresses the full length *oskar-MS2* mRNA, or that there is no truncation of the MCP and all the MCP is GFP tagged, or even that there are is no free GFP signal. Based on the specificity of *oskar* MBs, we propose that it is more likely that our nuclease-stable probes are reporting on transgenic *oskar* copies that are not labeled rather than giving non-specific signal. But it is beyond of the scope of this manuscript to analyze the MS2 system, which has been recently questioned and reported to yield non-specific effects. In yeast, it was shown that the genetic introduction of MS2 loops seems to produce decay intermediates that could possibly disrupt RNA metabolism and localization dynamics [Garcia and Parker *RNA*,. **21** (8), 1393-1395, (2015)].

We provided the requested percentage in REPRESENTATIVE RESULTS section, please also see Reviewer 1 comment #25.

27) *However, osk is packaged into RNPs containing many osk mRNAs. In all likelihood these RNPs do not discriminate between MS2-containing and -noncontaining mRNAs. Such co-packaging would explain why only 7% of MB particles do not contain MS2: the detectable MB particles are only those containing many copies of oskar. The authors should comment on this observation since it bears heavily on the question of detection efficiency.*

Please see our above response for *Reviewer 1 comment #26*. There is no endogenous *oskar* mRNA expression in the *oskar-MS2* transgenic ovaries, thus this explanation is unlikely. In addition, the GFP tag is known to mediate aggregation, which gives another reason why this transgene can induce artifacts.

28) *Discussion: Line 544 refers to "the kind and size [of] mRNA particle." osk RNPs are smaller than the diffraction limit. It is odd to discuss the size of particles whose physical size cannot be assayed with the methods presented. Please describe what size refers to in this context.*

In our lab we also employ superresolution microscopy of fixed samples, which we initially considered to include as supporting material for colocalization studies, however we decided it was beyond the scope of this manuscript. We modified the highlighted text and removed the mention of "size".

29) *In the table of materials, there is a listing of "Computer capabilities > 20 RAM" Presumably the units on 20 are GB, please modify for clarity.*

The information was corrected to show the unit.

Reviewer #2:

Manuscript Summary:
This manuscript describes protocols for designing and delivering molecular beacons (MBs) into Drosophila egg chambers and the subsequent imaging of hybridized MBs in vivo. Molecular beacons are the only available methodology for visualizing and tracking the dynamic behavior of endogenous mRNA transcripts in real time. However, the difficulties associated with designing molecular beacon probes have prevented more cell biologist from using this tool. In response, the Bratu lab has developed a new software application, PinMol, that uses the target mRNA sequence and secondary structure to efficiently design sensitive and specific molecular beacons.

Major Concerns:

Introduction:
The Introduction is most/more appropriate for a primary research article written for experts familiar with molecular beacons. However, it should be written for researchers who are not familiar with MBs, but are interested in potentially using them. I kept this in mind when reading/reviewing the manuscript. What would a researcher new to beacons want to know.

The focus of this manuscript is using MBs to visualize transport of endogenous mRNA. The editor mentioned from the beginning to stay away from computational/software description and present experimental results showing detection and tracking of endogenous mRNA. Please also see our response to *Reviewer 1 comment #3*.

1) The protocol would appeal to a broader audience if the introduction focused more on the structural and functional design of MBs and their general applications and less on Drosophila egg chambers.

This is not possible, as the manuscript with the detailed description of MB design using *PinMol* is freely available on *biorχiv*, and is currently under review at the *RNA* Journal. In addition, the JoVE team asked us to focus on our experimental expertise rather than the computational design of MBs.

2) Some suggestions for broadening the appeal of the protocol introduction and making it a more useful resource:
1. Change the title to: Visualizing and Tracking Endogenous RNAs Using Molecular Beacons

We believe it is important for the readers to know that our protocol was developed using fruit fly egg chambers, and we would like to keep the title as is.

2. Discuss the following information in the Introduction:

a. MBs have been used to detect mRNA. Have they been or could they be used to detect non-coding RNA?

We included the requested information in the INTRODUCTION section:

"Several groups have had success in using MBs to detect both non-coding RNAs (microRNAs and lncRNAs)[10-13], 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]."

b. What are some examples of tissue/cell types analyzed with molecular beacons?

We included the requested information in the INTRODUCTION section, see our response for the above comment (2a).

c. What factors should be considered when someone wants to begin using molecular beacons?
i. How do copy number & alternative splicing affect the usefulness of molecular beacons?

We already discussed in the manuscript how to improve visualization of low abundance targets, and this information is included in the DISCUSSION section, as we were guided by the editor.

"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, the 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. "

ii. What are the limitations of molecular beacons? Are some RNAs poor targets for MBs? If yes, why?

"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]."

iii. In general, what type of microscope set-up is needed to visualize MBs. How fast must they be? Can a standard confocal be used or is a spinning disc confocal required?

This depends on the events needed to be captured, we now provide acquisition parameters for the dataset presented in Fig. 4, please also see our response for *Reviewer 1 comment #16*.

d. A brief summary/overview of the procedure would be helpful to the researcher who has not worked

with beacons. In other words, what are the key procedures involved in using molecular beacons. 1. Design MB, 2. MB synthesis, purification & characterization, 3. Introduce MB into cell, 4. Image intracellular MB, 5. MB spot detection and tracking.

We believe that the numbered protocol steps already provide this information.

3) Line 133: The authors write "Upon image acquisition, it is essential that subsequent analysis steps are also completed carefully and precisely." This statement is too nonspecific and uninformative. It would be more useful to know why the image is processed and how it is analyzed. In other words, what purposes do these processes/software serve?

This is detailed within the PROTOCOL section, and this text has been moved from the INTRODUCTION to the DISCUSSION to address Editorial comment #10.

4) What controls are done? If you see a signal, how do you know that you are detecting the desired target transcript?

The specificity of *oskar* molecular beacons is well documented, and the manuscript has been edited to include details and the relevant references. Please also see our response for *Reviewer 1 comment #22*.

5) Line 478-484: Figure 1 legend does not match Figure 1. e.g. - (A) in Figure 1 shows a mid-stage egg chamber, however (A) in the figure legend states "Secondary structure region within oskar mRNA targeted by a molecular beacon."

Please see Editorial comment #3.

Minor Concerns:

Introduction:
6) Lines 46-47: The aptamer discussion in the long abstract is ambiguous and confusing and distracts from the discussion of molecular beacons. The statement "extensive genetic manipulation of the tissue") is too vague. This statement needs to be more precise (similar to line 77). I recommend first discussing the efficacy of molecular beacons and following this up with a comparison of MBs and aptamers.

This text has been deleted from the LONG ABSTRACT, please see our response to Editorial comment #6.

7) Line 84/85: The authors write "A technology that addresses this concern and offers additional unique advantages is the nucleic acid-based approach. Nucleic acid-based tool would be more accurate.

The text was revised as recommended.

8) Line 95: The authors write "The biggest drawback of the approach has been the design of MBs for live

cell imaging." "Has been the design of MBs" is ambiguous. Are the authors deferring to the act of manually designing the beacons or their structural design. I believe they are referring to the former, however this is open to interpretation.

The manuscript was edited for clarification. This text is now part of the DISCUSSION section.

9) Line 101: The authors write "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." Does PinMol eliminate these or is this something the researcher needs to do? As state, it is nor clear.

To clarify we added the following sentence:

"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."

Protocol:

1. Design of MBs for live cell imaging.

Line 156-157: "adjust the upper bound of number of computed foldings accordingly" does not make sense

We replaced "upper bound of the number" with "maximum number".

Line 167: Why does the researcher need to determine the specificity of selected MBs? What is this information used for? Are you looking for off target hybridization? This is important information for someone new to this method, which is presumably why they would be using this protocol.

First, there seems that the line numbering in the reviewers' copy differs from the copy we received. Line 171 mentions the specificity of the probes. This refers to possible cross-homology with other mRNA expressed in the tissue of interest. We added details to clarify that this sample and target-dependent step should be performed to ensure that other mRNAs do contain site that show high-complementarity with the selected probe sequence. Please also see our response to *Reviewer 1 comment #8*.

2. MB synthesis, purification and characterization

Lines 171-173: Why does the researcher need to determine the tissue specific expression of the target RNA? What is this information used for?

Please see our response to *Reviewer 1 comment #8*.

Line 175: How does someone select the appropriate fluorophore/quencher pair? Can you recommend a website/reference?

The fluorophore/quencher pair is dependent on the set-up available (*e.g.* wavelength of lasers for optimally exciting the fluorophore, emission filters). We added the relevant reference.

Lines 192-200: The authors recommend synthesizing one to five MBs. What do they recommend for someone new to MBs? Does the number depend on the size of the target RNA?

More MBs will enhance detection of low abundance transcripts, and this is addressed in the DISCUSSION section.

Lines 196-200: This section is verbose and confusing. Simplify and clarify

Since the line numbers do not correspond with our version, we assume this comment refers to lines 200-204. We modified this as follows:

"2.2. 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, Note 3). 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."

Lines 202-204: Why does the researcher perform thermal denaturation?

Lines 206-208, please see our response for *Reviewer 1 comment #12*.

3. Dissection and preparation of individual egg chambers

Line 211: Should emphasize that flies should be relatively young. Young flies produce more egg chambers.

We mentioned in the original manuscript to use 2-3 day old flies, which means that we collect the newly hatched flies and age them (while being well fed) for 2-3 days, thus providing an optimal number of mid-oogenesis egg chambers.

4. Microinjection of MBs into nurse cells of egg chambers

Lines 228-232: Confusing because sentence is too long and contains too much information. Split info into 2 sentences

The sentence was edited as follows:

"4.1. 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."

Line 243: Specify type of needle

Included in Table of Materials?

Yes, the information is included in the Table of Material, and we added a reference in the text.

Discussion:

Lines 537-542: The authors discuss post-acquisition processing of images. This section describes the potential uses of molecular beacons and would be useful in the introduction.

We included examples and references for usage of MBs to detect RNA targets in other organisms and tissues in the INTRODUCTION section, please see our response to *Reviewer 2 comment #2*.

Reviewer #3:

Manuscript Summary:
In their manuscript entitled "Visualizing and tracking Endogenous mRNAs in live Drosophila melanogaster egg chambers" Bratu at al describe a protocol that allows detection and tracking of mRNAs in live Drosophila tissue. This is an important protocol that could to be easily adopted to image non-genetically modified transcripts in living fly tissue. While all major points of the protocol seem to be covered the authors should better describe for each section the overall aim of the section as these are not clearly described. They should also better discuss the expected outcomes, potential problems and how to troubleshoot them. Where possible the authors should also discuss appropriate controls (see below in red and individual points discussed). Currently the manuscripts is written in such a way that it is not clear whether the protocol is set up to detect and track transport of oskar mRNA specifically using given MBs or that it could be adapted to track other transcripts. For these reasons the authors should spend more time defining the purpose and expected outcomes of each section and where possible provide and discuss additional controls. These are as follows:
Some questions to consider when reviewing the manuscript (provided by JoVe) (a detailed explanation for each point is provided below):
** Are the title and abstract appropriate for this methods article? YES*
** Are there any other potential applications for the method/protocol the authors could discuss? The*

authors could also state that this same protocol could also be used to detect mRNAs in fixed tissue. If so, please provide a quick description.
** Are all the materials and equipment needed listed in the table? (Please note that any basic lab materials or equipment do not need to be listed, e.g. pipettes.) NO (see point 6)*
** Do you think the steps listed in the procedure would lead to the described outcome? MOST LIKELY*
** Are the steps listed in the procedure clearly explained? NO. (see point 1,2).*
** Are any important steps missing from the procedure? YES (see point 3)*
** Are appropriate controls suggested? NO (see point 4, 5)*
** Are all the critical steps highlighted? NO (see point 3)*
** Is there any additional information that would be useful to include? YES (see point 3,4,5)*
** Are the anticipated results reasonable, and if so, are they useful to readers? YES*
** Are any important references missing and are the included references useful? NO*

Major Concerns:
Point 1 (see also point 2): For each section, the overall aim of the section is not clearly described, the expected outcomes are not discussed, potential problems and how to troubleshoot them are not discussed and in some cases the appropriate controls are not described.

This comment is broad, and we hope that by addressing the comments from the previous two reviewers we also touched on the points that the 3rd Reviewer wants us to address. We included discussion of critical steps and troubleshooting in the DISCUSSION section, please also see our response to the Editorial comment #10.

Point 2: Certain steps seem not to have relevance to the overall protocol or the relevance is not clearly stated. It should be clearly stated in each section what are the expected outcomes with appropriate controls. For example, the relevance of step 1.2.2 is not clear. How does filtering by for example tissue specificity contribute towards MB specificity/selectivity?

Please see *Reviewer 1 comment #8*.

In Step 4.3., why is it important to orient the sample in the A-P axis?

This is done to allow easy injection of nurse cells proximal to the oocyte, this info was included in the step description.

In step 5.2, why is imaging in this order necessary (better) as opposed to imaging each Z stack in its entirety before changing channels?

This is done for colocalization analysis purposes. If the Z-stack is first imaged in one channel and then in the second one, it will be difficult to assess colocalization between the two channels, as the positions of particles in the second channel have changed significantly by the time the first Z-stack acquisition is completed. We edited the end of this step to include "…, to allow for proper co-localization analysis.".

Why are steps 6.2.2. - 6.2.4. and 6.2.13-6.2.16. relevant to the protocol?

It is not clear what the reviewer is suggesting, removing the steps or adding more details? We included these steps to provide the user with the tools to produce image files appropriately labeled, and we believe they contain the necessary details.

In general, steps in section "Spot Detection and Tracking" are poorly explained as to why they are important and what choosing each of the specified commands does to the spot detection.

The outcome of the sub-steps is described in the title of the main step title, such as “Spot detection and Tracking”.

What is the expected outcome, how should images be thresholded,…what happens after each of these steps is completed? What does spot sensitivity mean (6.2.5.), what do "NoFiltering" or "Range of accepted objects" options perform (6.2.6.5.), what is "Pre Processing" in 6.2.6.2, what is "Relevant track processors" in 6.2.9,

We added some details in the protocol, which we hope address this comment.

what is sub-optimality and why would one choose 5-10% sub-optimality (step 1.1.1.)

Please see our response to *Reviewer 1 comments #4-6*.

What is a "Substack" and why is it needed (step 6.1.3).

A substack is a selection of a range of Z steps and time points to be further analyzed, the info was included in step 6.1.3..

What is the purpose of the co-localization analysis steps? Perhaps as a control that the correct signal was thresholded but in this sense one does not need to track the spots in live tissue.

Of course, colocalization analysis is not required nor possible when only one /fluorophore/color is visualized.

Point 3: Better describe what MBs are. How long should the stem be, how long the hairpin, what should be the GC content, what kind of fluorophore pairs are most suitable to make an MB. This last point is not even discussed though the authors mention several times that one can perform multicolor imaging with MB.

This is beyond the purpose of this manuscript. Please see our response to *Reviewer 1 comment #1* and *Reviewer 2 comment #1*. It would be really helpful if the guidelines provided to us when we were invited to contribute our work would be also provided to the reviewers.

Create a table outlining the best/most optimal fluorophore/quencher pairs. Currently, only two such examples are given (cy3/BHQ2 or cy5/BHQ2). Provide more examples of good quenchers with their quenching properties so readers can have a choice while designing MBs.

Appropriate references have been provided.

Equally importan, most of us will not attempt an in house MB synthesis/labeling. Provide suitable commercial providers that could synthesize MBs for us.

This is not allowed according to the instructions provided by the JoVE team.

Point 4: What is the purpose of the "in vitro" characterization of MB binding (page 5, point 2.1.-3)? This control seems superfluous since the in vivo controls are more relevant. If the authors deem this control important, they also need to describe it better (not clear as currently written).

Please see our response to previous comments: *Reviewer 1 comments #1, 12, and 18*.

Additionally, on page 11, line 453, the authors state that older MBs can give rise to non-specific signal but that the MBs designed with a new approach do not. Please explain why, specifically since the MBs were always considered as the probes that have no fluorescence unless bound to a target RNA.

This is already known and we have discussed it as related to the presented protocol in our response to *Reviewer 1 comments #1 and 2*.

Point 5: Appropriate controls are not discussed. For example, how many MBs would one need to reliably detect a single mRNA either in fixed or in live tissue? How is threshold for detection of MB-labeled mRNAs set (it seems to vary depending on tissue, which needs to be explained why). Additionally, how do you control for accurate tracking of moving spots - the authors must discuss how to set up the parameters for correct imaging speed to allow recording of moving mRNAs and their trajectories (imaging too slow and only slow moving transcripts will be detected, while imaging in 3D might capture rapid events in 3D but will increase the bleach rate). All these aspects which will critically influence the outcome of the experiments have not been discussed.

We previously characterized *oskar* mRNA trafficking in the egg chamber [Mhlanga, M. M. *et al. PLoS One.* **4** (7), e6241, (2009)]. The acquisition parameters, including the sampling rate, are highly dependent on the organism and tissue used, on the temporal and spatial gene expression and target mRNA trafficking, as well as on the microscope set-up. Our protocol is mainly focused on the analysis, but as an example, at the request of Reviewer 1, we added the acquisition parameters used for the data presented in Fig. 4 (see response for Reviewer 1 comment #16).

Point 6: Please discuss in better detail the microscope/detector/ resolution that will allow appropriate imaging and tracking of mRNAs in live tissue. Also briefly describe the injection rig setup (model, provider,…).

Please see our response for *Reviewer 1 comment #16 and 19*, and point 5 above.

Point 7: Better explain why the co-localization measurements are needed to achieve tracking of moving

mRNAs. Are there other co-localization methods available that would be appropriate substitutes or is the one provided by the authors the only acceptable one (and why). Additionally, the authors detect a 4 pixel (define size of a pixel) distance between co-localizing spots labeled with spectrally distinct colors (page 12, line 501). Is this a pixel shift?

Colocalization is not needed for the purpose of tracking, the mRNA alone can be tracked. In this protocol we described object-based colocalization to illustrate how one can determine the degree of colocalization between an mRNA and a protein of interest. We chose oskar-MS2 to show the overlap between the two detection methods, but each MB and GFP can be tracked separately without performing colocalization analysis. Other colocalization methods have been used for mRNA localization in fixed *D. melanogaster* embryo. However, for tracking purposes we need to use object-based colocalization. This information/reference is now included in the DISCUSSION section.:

"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]"

The size of pixel is now included for the objective used for acquisition (legend of Fig. 4).

Minor Concerns:
Minor points:
- Number 1.1. in line 162 should be 1.1.2.

In our version the step mentioned appears to be in order 1>1.1.>1.1.1.>1.1.2.l appear in order.

- In point 3.1., define the age range of females.

We clarified this information in the text.

- Point 4 (line 226). It seems important to inject MBs into nurse cells and not in nurse cell nuclei or oocytes. Why?

Not true, you can inject in the oocyte and Fig. 3 shows side-by-side data obtained from microinjections performed in the nurse cell (Fig. 3A) and oocyte (Fig. 3B). However,injecting the MB into the nurse cell enables us to track mRNA particles in both the nurse cell cytoplasm and ooplasm, as well as visualize mRNA localization in the oocyte.

- Why do the authors prefer an angle smaller than 45 degrees during the injection (line 244).

To make sure you don't puncture several nurse cells, and the info was included in the text.

- A step is missing (or is it) after 4.11. where the needle is removed from sample?

We included an additional step:

"4.12. Gently remove the needle and retract it to the home position."

- Define "XYZCt"

This info was included in step 5.1.

- …30 min time points should perhaps be "min after MB injection"? (line 487)

We added "…, after initiation of acquisition". in the legend of Fig. 2.

- Are the measurements performed every 30 sec for 20 min in Z (line 497)?

This information is included in the legend of Fig. 4:

"XY-projections of 14 Z optical slices at 0.3 µm steps.".