**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 cells24. *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 signal34, 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 signal33,36.”

1. *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 signal34, 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 signal33,36.”

Comments follow:

1. *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.

1. *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*).

1. *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.

1. *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.

1. *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.”

1. *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.”

1. *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.

1. *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 oligonucleotides8.”

1. *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 described7, 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.”

1. *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 described7. 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 described7. 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.

1. *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).”

1. *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.

1. *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.

1. *-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.

1. *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.

1. *-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.”

1. *-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 oocyte23. 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.

1. *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.

1. *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 oocyte23. 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.”

1. *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 approach8, and MB co-injection with *in vitro* transcribed *oskar* mRNA labeled with a fluorophore spectrally distinct from the MB’s label23.”

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

Please see Editorial comment #4.

1. *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.

1. *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.”

1. *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.

1. *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.

1. *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”.

1. *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 retroviruses14 and dynamic DNA-protein interactions15. They have been successfully employed for imaging in various organisms and tissues, such as zebrafish embryos16, neurons13, tumor tissue17, differentiating cardiomyocytes18, 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 probe31.”

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 MgCl2 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.”.