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Intracellular single molecule microscopy reveals two distinct pathways for microRNA assembly

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1st Editorial Decision

06 December 2011

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all the referees find the topic of interest, they also consider the study preliminary for publication here at this stage. All of them request numerous technical improvements of the data, and point out that further experiments are needed to provide convincing support for the identification of the particles, the colocalization with mRNA targets and to strengthen your claim of a multiple target turnover model.

From the analysis of these comments it is clear that publication of your manuscript in our journal cannot be considered at this stage. On the other hand, given that all referees provide constructive suggestions on how to make the work more conclusive, I would like to give you the opportunity to revise your manuscript. From the reports pasted below and further discussions with the referees, it is clear that a lot of new experimentation would be required and it is uncertain whether these results would back up your current claims. In particular, it would be crucial to demonstrate that the observable particles contain miRNAs bound to and repressing their mRNA targets, to identify RISC and PBs by co-staining (or alternative procedures), experimentally strengthen the support for the multiple turnover model, and address all other major points of the three referees, which are in broad agreement. Please note that it is EMBO reports policy to undergo one round of revision only and

thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

In addition, I have noted that there is an extensive supplementary discussion and supplementary references section, which we do not allow, and some of the most relevant methods are also in the supplement. Please ensure that Materials and Methods required for understanding the experiments performed must remain in the main text, although more detailed explanations necessary to reproduce them can be included in the supplement.

Should you decide to embark on such a revision, revised manuscripts should be submitted within three months of a request for revision unless previously discussed with the editor; they will otherwise be treated as new submissions. I could potentially further extend this period (if time were the only limitation to a successful revision of the paper) but would also understand your decision if you rather chose to seek rapid publication elsewhere at this stage.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance. Should you decide to submit your paper elsewhere, I would welcome a message to that effect.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The report by Pitchiaya et al. describes a method to study miRNA diffusion and assembly into functional ribonucleoprotein (RNP) complexes by a combination of single particle tracking (SPT) and fluorophore photobleaching step counting in live and fixed cell, respectively. The introduction of organic dye labeled miRNA into cell by microinjection, as well as highly inclined laminar optical sheet (HILO) microscopy, significantly reduce the background fluorescence and enable single molecule detection in vivo. This method allows tracking of individual miRNA diffusion upon microinjection and putative assembly of miRNAs into RNP complexes or P-bodies (but more controls are needed to establish these claims, see below). If established, the method provides a generalizable approach to investigate the cellular behavior of miRNAs including both diffusive properties and assembly states of functional intermediates. But before further consideration in EMBO reports, there are several technical issues that need to be addressed through controls, as listed below.

1. Microinjected vs. endogenous miRNAs

If I understand correctly, the labeled let-7a-1 miRNAs microinjected for SPT and photobleaching step counting are wild type. Based on the results of luciferase reporter assay, even without exogenous let-7a-1, endogenous let-7a-1 level is high enough to repress the target sequence. In this case, labeled and endogenous miRNA will compete for the assembly of RNPs. It will not affect the results and interpretation for tracking experiments, however, this situation should be taken into consideration when counting the number of photobleaching steps for the particles and drawing any conclusion based on the population percentage of particles containing single vs. multiple labeled miRNAs; for example, how many of the particles containing single labeled miRNA actually really contain single miRNA? The authors should have a discussion on this issue and also perform some control experiments: for example, 1) titrating the concentration of labeled miRNA microinjected and checking if the population percentages of particles containing single vs. multiple labeled miRNAs change with the concentration of labeled miRNA; 2) using the labeled mutant let-7a-1 miRNA with the mutant targeting sequence, as in the luciferase reporter experiments, to serve as a control in which endogenous wild type let-7a-1 will not be able to interfere.

2. Controls using mutant let-7 will be important to establish that the observed fluorescent spots are

actually related to the miRNA function as proposed in Fig. 4. If their interpretation is correct and the observed spots are indeed functional important complexes for miRNA-based repression, dynamics and assembly states of the spots should be dramatically different for the mutant *let-7*.

3. Fast vs. slow diffusion and single vs. multiple miRNAs

SPT reveals that the miRNA containing particles have two distinct diffusion constants, which closely resemble those of messenger RNPs and processing bodies (PBs), respectively. In the photobleaching step counting experiment, miRNA containing particles are categorized into the ones with single miRNA and with multiple miRNAs. The time-dependent change of relative populations of these two classes suggests the existence of two kinetic processes, which by the authors, interpreted as the assembly of miRNA and/or miRNA RISC (containing single miRNA) into functional RNPs (including both messenger RNPs and PBs, and containing multiple miRNAs) and the disassembly of RNPs after mRNA degradation. Since the latter experiment has been performed in fixed cell, single miRNA or miRNA RISC can be well detected, while they are not resolvable in SPT experiment because of their fast diffusive property and the time resolution. In this scenario, a much larger fraction of particles containing multiple miRNAs in SPT experiment should be expected compared to the photobleaching step counting experiment. However, the authors have observed a strong overlap in the distribution of fluorescence intensities (an indication of number of fluorophores) between particles in these two experiments (supplementary fig 8), which seems to be self-contradictory.

4. Sensitivity of the method

The time-dependent change in the fraction of single and multiple miRNA containing particles observed in *let-7a-1* is not observed in *cxcr4* miRNA, and the authors attribute this to the fact that *cxcr4* has 10-fold fewer mRNA targets, therefore the change in population in this case will be too small to be detected. This interpretation needs to be tested by cutting down the amount of *cxcr4* microinjected. In addition, based on the observation that the "sensitivity" of the method seems to be dependent on the target level of a specific miRNA, maybe the authors can comment on what is the lower limit of the target level for a particular miRNA when applying this method to study its assembly into functional states.

Referee #2:

In this manuscript, Pitchiaya et al. report a method to track microinjected, fluorescently labeled miRNAs in cells. Based on the observations, the authors propose a model of multiple target turnover by miRNAs. While interesting, there is no direct evidence supporting the authors' argument, and there are a number of additional control experiments to be performed before the manuscript is being considered further.

Major points:

1. There are many previous reports for intracellular single molecule tracking of fluorescently labeled RNAs (e.g., Shav-Tal et al. *Science* 2004; Tadakuma et al. *BBRC* 2006; Ishihama & Funatsu *BBRC* 2009; Grunwald & Singer *Nature* 2010), and the novelty of iSHiRLoC is unclear. The authors should more carefully highlight or tone down the originality of the method.
2. Silencing efficiency in Fig. 1 should be shown as either raw measured values (most preferred), or "fold repression" normalized to the value of control siRNA. Arbitrarily normalizing the values to that of luc siRNA as "% repression" is unusual and inappropriate. Directly comparing different constructs (i.e., wt vs. mut target plasmid constructs) is also inappropriate, because their transcription/translation efficiencies are not warranted to be identical. Moreover, the authors should validate the specificity of this experiment by blocking wt and mut *let-7-a1* by respectively complementary 2'-O-Me antisense oligonucleotides.
3. There are no direct evidence that the observed populations correspond to target-bound RISC and PBs. Without experimental validation, it is impossible to argue any model merely by comparing the diffusion coefficients with those previously reported and tracking the changes of populations over time. The authors must confirm co-localization of injected miRNAs and Argonaute proteins and PB

markers (suitable antibodies should be readily available) in fixed samples over time. Tracking should also be performed after RNAi-mediated depletion of Argonaute or PB components. Moreover, rather than merely comparing let-7 miRNA and CXCR4 small RNA, the authors should more directly verify the target-bound form, by examining co-localization of microinjected miRNA and its target mRNA (e.g., by FISH and/or by utilizing a fluorescent protein and RNA-binding motifs such as MS2, lambdaN, L7Ae etc.). In this case, 2'-O-Me antisense oligonucleotides can be utilized to specifically block target binding.

4. The proposed multiple turnover model lacks any concrete evidence. As stated above, experimental validation of observed populations is mandatory. Also, it is unclear if the "changes" in Fig. 3E and Supplementary Fig. S9 are really statistically significant. Moreover, if the proposed model (based on Fig. 3E) is true, then the "fast" complex ($0.26 \mu\text{m}^2/\text{s}$; RNP or target-bound RISC) should increase at later time points in Fig. 2G, but it is apparently not the case.

Minor points:

1. The first line in introduction: "RNA interference (RNAi)" should read "RNA silencing". These two words used to be used as very similar meanings, but RNAi now specifically refers to phenomena that accompany cleavage of complementary targets.
2. The labels and legend of Fig 1 are very confusing. What do "-ve" and "Ambion-vectrl" stand for? What's the difference between the second and third samples? Probably the third sample was with the mut target plasmid, not wt.
3. Supplementary Fig. S9 should show the assayed times (20 min to 32 h) as in Fig. 2G. Also, 32 h is longer than the cell division time and should thereby be avoided.

Referee #3:

In this manuscript, the authors attempt to understand aspects of miRNA biology by injecting dye-labeled miRNA into the cell and observing their dynamics at the resolution of individual molecules. By these means, they observed diffusing particles that separate into slow-diffusing and fast-diffusing subpopulations, which they believe correspond to various different miRNA species. By watching how these populations change over time after microinjection, the authors can make statements about the dynamics of various processes involved in miRNA processing.

Overall, I think this paper is technically solid and provides important data for constructing mechanistic models of miRNA function. Their method appears relatively accessible to other researchers as well, which I think increases the potential impact of the work. Most of the controls seem relevant and informative, and their approach seems to be sound.

My one major issue is one of interpretation. The authors observe two populations of spots: one with a diffusion coefficient of around $.26 \mu\text{m}^2/\text{s}$ and one with a diffusion coefficient of around $0.034 \mu\text{m}^2/\text{s}$. They claim that the first population is most likely miRNA/target RNP complexes, whereas the second population corresponds to miRNAs in P-bodies. As far as I can tell, in their view, the hierarchy is:

miRNA: too fast to detect
 miRNA-AGO: too fast to detect
 miRNA-AGO-RNP: $D=0.26$
 miRNA-AGO-RNP-P-body: $D = 0.034$

Based on other literature from other groups (Golding et al. PNAS 2004, Shav-Tal et al. Science 2004, Vargas et al. PNAS 2005), it seems that the most likely that mRNP diffusion coefficients are closer to the 0.034 value than 0.26. Moreover, the nature and dynamics of P-bodies is somewhat mysterious and certainly the case for miRNA/P-body associations is a relatively new one that has not yet been fully characterized. One alternative hierarchy could then be:

miRNA: too fast to detect

miRNA-AGO: D=0.26
 miRNA-AGP-RNP: D=0.034
 miRNA-AGO-RNP-P-body: undetectable (for other reasons)

I think the authors need to perform some experiments in order to distinguish between these possibilities. For instance, a co-stain with well-known P-body markers (either fused to GFP in live cells or IF in fixed cells) would be sufficient to show that the fraction they claim to be in P-bodies are actually in P-bodies (I realize that Pillai 2005 did some sort of demonstration of this, but I think an independent assessment would be wise considering that these labeled miRNAs may behave differently). Perhaps an AGO/RISC GFP fusion might also shed some light on the nature of the fast-moving particles the authors observe. Another thing the authors could do is perform single molecule FISH on the target after fixation to see what fraction of any particular target is bound to miRNA.

The authors might argue that they do see a population that requires multiple steps to photobleach, which perhaps they would assume to then be P-bodies. However, I'm assuming many of the endogenous targets for these particular miRNAs in single cells have multiple miRNA binding sites per mRNA, in which case, one might see multiple photobleaching steps for labeled miRNAs when they are bound to the target mRNP even if they are not in P-bodies. I think the authors should certainly at least examine the number of potential targets for which there are multiple miRNA binding sites.

Other, relatively less major issues:

-The authors say that cells fixed after just 20 minutes have high levels of background. This would imply that the number of labeled miRNAs decreases over time, suggesting that there is either some degradation (and subsequent ejection of the dye) or that the cell somehow expels the labeled miRNAs. In either case, this means that the authors' estimate of the number of miRNAs they microinject may not be accurate. Can the authors comment on this point, and more generally, on the degradation/export properties of their probes?

-The figures are rather confusing to parse, with few indicative labels on the graphs. I suggest considering ways to make the figures easier to understand. For example:

-Figure 1a has a variety of graphs labeled with things like -ve*, WT*, etc., that are perhaps understandable to those in the miRNA field, but are somewhat confusing to those outside it. Perhaps a diagram?

-Figure 1b is very hard to interpret without delving into the legends and text.

-Figure 3E has no indication on what the dotted lines and solid lines represent.

Again, these are merely stylistic suggestions-nothing in the graphs is misleading in any way.

In summary, I think this is a solid piece of work that could make a real contribution to the miRNA field, as well as linking that field to the biophysics field. My main concern is about the interpretation of the identities of the spots they detect, and I would have to see some conclusive evidence to support their claims before recommending publication.

In the following, please find our detailed point-by-point responses to the reviewers' suggestions and concerns.

Referee #1: This reviewer positively notes– “If established, the method provides a generalizable approach to investigate the cellular behavior of miRNAs including both diffusive properties and assembly states of functional intermediates”. H/She also points to several technical issues that ought to be addressed, which we did as follows.

1. *Microinjected vs. endogenous miRNAs.* If I understand correctly, the labeled *let-7a-1* miRNAs microinjected for SPT and photobleaching step counting are wild type. Based on the results of luciferase reporter assay, even without exogenous *let-7a-1*, endogenous *let-7a-1* level is high enough to repress the target sequence. In this case, labeled and endogenous miRNA will compete for the assembly of RNPs. It will not affect the results and interpretation for tracking experiments, however, this situation should be taken into consideration when counting the number of photobleaching steps for the particles and drawing any conclusion based on the population percentage of particles containing single vs. multiple labeled miRNAs; for example, how many of the particles containing single labeled miRNA actually really contain single miRNA? The authors should have a discussion on this issue and also perform some control experiments: for example, 1) titrating the concentration of labeled miRNA microinjected and checking if the population percentages of particles containing single vs. multiple labeled miRNAs change with the concentration of labeled miRNA; 2) using the labeled mutant *let-7a-1* miRNA with the mutant targeting sequence, as in the luciferase reporter experiments, to serve as a control in which endogenous wild type *let-7a-1* will not be able to interfere.

The reviewer is correct that we are only reporting on the microinjected fluorophore labeled miRNAs, and that this fact does not affect our results in any way. A single fluorophore labeled miRNP particle will in fact likely contain also endogenous, unlabeled miRNAs including wild-type *let-7*, but we never intended to claim otherwise. To avoid confusing the reader, we have made the following simple adjustments to the text:

Abstract:

“Microinjected, singly-fluorophore labeled, functional miRNAs were tracked within diffusing particles, a majority of which contained single **such** miRNA molecules.”

Page 8:

“...While close to 50% of all particles contained single **fluorophore labeled** miRNAs 2 h after microinjection, a significant fraction held up to seven **labeled** miRNAs.”

As for the suggested controls:

1) We know that these experiments are concentration sensitive and titration of labeled miRNAs, especially to lower concentrations, will merely further move the distribution to single photobleaching steps, as the probability of encountering a particle with two or more fluorescent probes will decrease. Again, since we do not claim to count the number of ALL, but only that of fluorophore labeled miRNAs, there is really no point to this experiment in the context of the current manuscript. The number of microinjected *let-7* miRNAs is similar to that already present endogeneously, hence one would expect about equal probability to encounter a labeled and unlabeled *let-7* miRNA molecule in any given complex. Since all our conclusions only depend on the changes over time of the relative (not absolute) numbers of a given miRNA assembled into RNPs and PBs, no absolute quantification of ALL miRNAs in such complexes (which would be very challenging at best) is necessary.

2) As suggested, we have now added new data (**Supplementary Fig. 2B**, discussed on page 7) showing colocalization of the mutant miRNA with the mutant target sequence, signifying functional assembly of microinjected miRNAs on their cognate mRNA targets.

2. *Controls using mutant *let-7* will be important to establish that the observed fluorescent spots are actually related to the miRNA function as proposed in Fig. 4. If their interpretation is correct and the observed spots are indeed functional important complexes for miRNA-based repression, dynamics and assembly states of the spots should be dramatically different for the mutant *let-7*.*

Essentially as suggested by the reviewer, we have now added control experiments with the artificial and particularly target-poor *cxcr4* miRNA, in lieu of the mutant *let-7* miRNA. As expected, the assembly states of *cxcr4* miRNA are indeed dramatically different from those of wild-type *let-7*, favoring monomers (**Fig. 3E**). Going even further, we observe a rescue of multimeric miRNA assembly when specific mRNA targets of the *cxcr4* miRNA are co-injected with the miRNA (**Supplementary Fig 7**), strongly suggesting that the observed spots are indeed functionally important for miRNA mediated mRNA repression. These new data are discussed on pages 10/11. Interestingly, the distribution of diffusion constants of *cxcr4*-miRNA containing particles is only mildly different from that of *let-7* miRNA (**Supplementary Fig.**

1C, D, E). This is a consequence of the fact that our particle tracking can only report diffusion coefficients of a fraction of all diffusing particles, i.e., those diffusing slowly enough to be visible in the focal plane for over 9 imaging frames (0.9 s). Please note that we added a brief discussion of our time resolution on page 7 of the revised manuscript (see also referee 3 below).

3. *Fast vs. slow diffusion and single vs. multiple miRNAs. SPT reveals that the miRNA containing particles have two distinct diffusion constants, which closely resemble those of messenger RNPs and processing bodies (PBs), respectively. In the photobleaching step counting experiment, miRNA containing particles are categorized into the ones with single miRNA and with multiple miRNAs. The time-dependent change of relative populations of these two classes suggests the existence of two kinetic processes, which by the authors, interpreted as the assembly of miRNA and/or miRNA RISC (containing single miRNA) into functional RNPs (including both messenger RNPs and PBs, and containing multiple miRNAs) and the disassembly of RNPs after mRNA degradation. Since the latter experiment has been performed in fixed cell, single miRNA or miRNA RISC can be well detected, while they are not resolvable in SPT experiment because of their fast diffusive property and the time resolution. In this scenario, a much larger fraction of particles containing multiple miRNAs in SPT experiment should be expected compared to the photobleaching step counting experiment. However, the authors have observed a strong overlap in the distribution of fluorescence intensities (an indication of number of fluorophores) between particles in these two experiments (supplementary fig 8), which seems to be self-contradictory.*

We appreciate the reviewer's keen observation. However, estimating the number of fluorophores per particle in live cells based on a particle's intensity, as the reviewer implicitly does here, is error prone due to the blurring effect caused by the particle's movement. Movement results in a distribution of fluorescence signal over a larger area (number of pixels) on the camera's CCD chip, thus lowering the fluorescence intensity detectable from a single particle. This effect does not occur for a fixed particle, as the reviewer points out. Consequently, the strong overlap in fluorescence distribution from individual particles in live and fixed cells is NOT self-contradictory as it is likely aided by a lowering of the fluorescence intensity in both cases, but through different effects – blurring in the case of live cells and higher chances to observe particles with fewer fluorophores in fixed cells. What this discussion shows is, however, that live and fixed cell experiments are nicely complementary and speak to different properties (diffusion and assembly state, respectively) of miRNA containing particles, as we emphasize throughout the manuscript.

We also note that we report our comparison of particle intensities at a stage (2 h after microinjection) where the 'slow' and 'fast' populations in live cells are almost equally distributed and where we observe a maximal change in the assembly of miRNAs (**Fig 3E**). Thus, it is highly likely that a predominant fraction of microinjected miRNAs at this stage has indeed assembled into large RNPs, slowing down their diffusion and further favoring the observed overlap in intensity distributions between live and fixed cells.

To clarify this latter point we have made the following changes to the revised manuscript:

Page 9:

"In addition, we **observed** a strong overlap in the distribution of fluorescence intensities between particles in fixed and living cells **2 h after microinjection (Supplementary Fig S5E), when most miRNAs are assembled into RNPs**, suggesting that our counting results in fixed cells **closely reflect** the miRNA assembly states in living cells."

4. *Sensitivity of the method. The time-dependent change in the fraction of single and multiple miRNA containing particles observed in let-7a-1 is not observed in cxcr4 miRNA, and the authors attribute this to the fact that cxcr4 has 10-fold fewer mRNA targets, therefore the change in population in this case will be too small to be detected. This interpretation needs to be tested by cutting down the amount of cxcr4 microinjected. In addition, based on the observation that the "sensitivity" of the method seems to be dependent on the target level of a specific miRNA, maybe the authors can comment on what is the lower limit of the target level for a particular miRNA when applying this method to study its assembly into functional states.*

Decreasing the level of microinjected cxcr4 miRNA will further bias the distribution towards monomeric miRNA particles in our fixed cell photobleaching analysis. Thus, we instead

performed an equivalent experiment, by co-microinjecting ~3,000 mRNA target molecules, each bearing six *cxcr4* binding sites, along with ~18,000 *cxcr4* miRNA (**Supplementary Fig 7**). Accordingly, we observed an increase in the number of particles containing multiple labeled miRNAs as compared to those where no mRNA or a control mRNA bearing no *cxcr4* binding sites was co-microinjected. Consequently, we have made the following changes to the main text:

Pages 10/11:

“Consistent with this model, cells microinjected with the artificial *cxcr4* miRNA, which is predicted to find 10-fold fewer mRNA target molecules in a HeLa cell (**Supplementary Methods**), do not show these time dependent changes in the fractions of single and multiple miRNA containing particles (**Fig 3E**; since the decrease in monomeric miRNAs is expected to be ~10-fold smaller for *cxcr4* than *let-7-a1*, it becomes indiscernible in our experiments with an estimated standard error of the mean of ~5-10%). In further support, co-microinjecting ~3,000 mRNA molecules targeted by *cxcr4*-miRNA increased the fraction of multiple miRNA containing particles at the expense of monomers, while co-microinjecting control mRNA molecules did not (**Supplementary Fig S7**).”

Referee #2: This reviewer finds our study “interesting”, but asks for additional comparisons and control experiments, which we provide as follows.

Major points:

1. *There are many previous reports for intracellular single molecule tracking of fluorescently labeled RNAs (e.g., Shav-Tal et al. Science 2004; Tadakuma et al. BBRC 2006; Ishihama & Funatsu BBRC 2009; Grunwald & Singer Nature 2010), and the novelty of iSHiRLoC is unclear. The authors should more carefully highlight or tone down the originality of the method.*

We concur with the referee that many reports for single molecule tracking of fluorescently labeled large RNAs exist. However, a large majority of these methods label the RNA either with multiple probes (Shav-Tal et al. Science 2004; Grunwald & Singer Nature 2010) or with large probes like quantum dots (Ishihama & Funatsu BBRC 2009), which artificially increase the molecular weight of the RNA and thereby lead to artificially slowed diffusion. Tadakuma et al. BBRC 2006 could detect mRNAs labeled with as little as two Cy3 probes, but used a non-specific labeling strategy that resulted in a heterogeneously labeled sample, with RNAs containing anywhere between 0 – 8 probes. In contrast, iSHiRLoC uses homogeneous, singly labeled (with small, organic fluorophores) miRNAs to track particles in live cells. Additionally, it deduces the assembly states of particles in fixed cells at single molecule sensitivity. Applying these techniques to small non-coding RNAs such as miRNAs is entirely novel. To further highlight the differences of iSHiRLoC from previous work, we have modified the introduction as follows (we also note that the strict length limits of *EMBO reports* clearly set limits to the extent to which we can elaborate in the text):

Pages 3/4:

“The main strategies for SPT in living cells either decorate the target molecule with multiple endogenously expressed fluorescent protein(s) (Grunwald *et al*, 2011) or endocytosed nanoparticles (Pinaud *et al*, 2010), leading to high-molecular weight appendices that may impede function; or they employ microinjection of target particles externally labeled with multiple fluorophores (Babcock *et al*, 2004). Complementary methods have recently been developed to assess the number and location of single intracellular molecules by fixing and permeabilizing a cell and incubating it with probes that are labeled with multiple fluorophores or nanoparticles (Itzkovitz and van Oudenaarden, 2011), limiting this approach to the detection of relatively large protein and RNA molecules. Our intracellular single molecule, high-resolution localization and counting (iSHiRLoC) method overcomes these caveats by combining microinjection, low-background illumination, SPT and stepwise photobleaching to visualize the diffusive motions and distribution of singly fluorophore labeled functional small RNAs inside cells. We observe two kinetically distinct pathways involving mRNA dependent miRNA assembly processes, providing a direct look at the complexity of the intracellular processes involved in RNA silencing.”

2. *Silencing efficiency in Fig. 1 should be shown as either raw measured values (most preferred), or "fold repression" normalized to the value of control siRNA. Arbitrarily normalizing the values to that of luc siRNA as "% repression" is unusual and inappropriate. Directly comparing different constructs (i.e., wt vs. mut target plasmid constructs) is also inappropriate, because their transcription/translation efficiencies are not warranted to be identical. Moreover, the authors should validate the specificity of this experiment by blocking wt and mut let-7-a1 by respectively complementary 2'-O-Me antisense oligonucleotides.*

We have modified the figure in question (**Fig 1a**) to avoid confusion and better conform to conventions in the field. The y-axis was changed from “% Repression” to the raw measured values, or “Relative Luciferase Activity”, normalized to the value for the negative control siRNA cotransfected with the mutant plasmid. The comparison between mut and wt plasmids had to be included to specifically show that a reporter with seven let-7 seed sequences was strongly repressed even without adding exogenous let-7-a1, presumably owing to the high abundance of endogenous let-7 in HeLa cells. This observation prompted us to use a mutant reporter and mutant let-7-a1 miRNA pair to show, with much higher sensitivity, that the attachment of a fluorescent probe to the 3'-end of the miRNA guide strand does not significantly affect repression.

The specificity of interaction between a miRNA and its cognate reporter gene is already clearly shown— Comparing the relative expression of the reporter in cells cotransfected with a negative control (i.e., non-specific) siRNA, a wild-type let-7-a1 miRNA, or a mutant let-7-a1 miRNA, we find that the mutant reporter is only repressed by the mutant let-7-a1 miRNA, whereas the wild-type reporter is only repressed by the wild-type let-7-a1 miRNA, as expected (compare samples a, b and c, with a*, b* and c* in the modified **Fig 1a**). Blocking of a miRNA with a complementary 2'-O-Me antisense oligonucleotide would therefore be redundant.

3. *There are no direct evidence that the observed populations correspond to target-bound RISC and PBs. Without experimental validation, it is impossible to argue any model merely by comparing the diffusion coefficients with those previously reported and tracking the changes of populations over time. The authors must confirm co-localization of injected miRNAs and Argonaute proteins and PB markers (suitable antibodies should be readily available) in fixed samples over time. Tracking should also be performed after RNAi-mediated depletion of Argonaute or PB components. Moreover, rather than merely comparing let-7 miRNA and CXCR4 small RNA, the authors should more directly verify the target-bound form, by examining co-localization of microinjected miRNA and its target mRNA (e.g., by FISH and/or by utilizing a fluorescent protein and RNA-binding motifs such as MS2, lambdaN, L7Ae etc.). In this case, 2'-O-Me antisense oligonucleotides can be utilized to specifically block target binding.*

As suggested, we have now added new data (**Supplementary Fig 2**) clearly showing the colocalization of microinjected miRNAs with both cognate mRNA targets and processing bodies (PBs), providing direct evidence for target and PB binding miRNA populations, respectively. The mutant let-7 miRNA and the mRNA bearing the corresponding mutant seed sequences with 24x MS2 binding sites for labeling with GFP was used in the former experiment to prevent interference from endogenous let-7. These data immediately and fully address the reviewer's concern that the observed miRNA populations indeed correspond to, at least in part, target-bound RISC and PBs.

We found it to be much more challenging to colocalize individual Ago proteins with single miRNAs since Ago proteins are so abundant (intracellular concentration = ~80 nM, Petri et al., RNA 2011, Vol. 17, p737-749) that visualizing them by immunohistochemistry results in broad fluorescence throughout the cell. Accumulation of signal within certain punctate structures has also been reported, but this corresponds to localization to stress granules and PBs (Leung et al, PNAS, Vol. 103, p18125-18130). This makes the visualization of the interactions of single miRNAs with Ago protein extremely difficult against a very high fluorescent background from free Ago and Ago bound to other cellular miRNAs (data not shown). For this purpose, higher temporal resolution and/or ultra-high resolution microscopy techniques such as STORM or PALM (Huang et al. Annu. Rev. Biochem., 2009, Vol. 78, p993-1016) are needed, which go well beyond the scope of the current report.

Finally, applying iSHiRLoC after RNAi-mediated depletion of Ago proteins and PB markers, although possible, requires careful titration and proper timing of siRNA addition to obtain the desired effects and therefore goes beyond the current report. In addition, these experiments may well result in unaccountable secondary effects. For instance, siRNA mediated depletion of Ago or PBs may be reversed by microinjecting miRNAs that compete for Ago protein. Overexpressing dominant negative forms of Ago and PB markers is also possible, but the high abundance of the endogenous proteins may convolve the data. Both of these experiments are still plausible controls, but we feel are redundant in light of the extensive colocalization data already added to the revised manuscript.

4. *The proposed multiple turnover model lacks any concrete evidence. As stated above, experimental validation of observed populations is mandatory.*

Previous reports (Hutvagner et al., Science, 2002, Vol. 297, p2056-2060; Anderson et al., Nature Rev. Mol. Cell. Biol., 2009, Vol. 10, p430-436) have already proposed multiple substrate turnover by miRISC complexes and other cellular RNA degradation machinery, consistent with our model. Additionally, the time frame of assembly/disassembly in our experiments closely matches that reported for miRNA mediated translational repression (Fabian et al., Mol. Cell, 2009) and mRNA degradation (Guo et al., Nature, 2009), and depict two different kinetic processes consistent with a multiple-turnover model. However, as our evidence is only indirect (although further corroborated by colocalization of microinjected miRNAs with target mRNAs and PBs, see point 3 above), we have made the following changes to our text to further accommodate this concern:

Refocused Abstract:

“Observed mobility and mRNA dependent assembly changes suggest the existence of two kinetically distinct pathways involving miRNAs, revealing the dynamic nature of an important gene regulatory pathway and paving the way towards its single molecule systems biology.”

Page 10:

“Based on these observations, we propose the following time-dependent model for miRNA assembly: At initial time points, such as 20 min and up to 1 h (Supplementary Fig S6A and Supplementary Fig S3A) after microinjection, a significant fraction of our miRNAs remains free, thus contributing to a large background in our live and fixed cell experiments. Between 1 and 2 h, most miRNAs have assembled into miRISC-mRNA complexes, thereby depleting the free miRNA population. This assembly leads to relatively slowly diffusing complexes in living cells and a corresponding decrease in the population of single miRNAs containing particles in fixed cells. Still later, miRNAs mediate mRNA degradation via association with PBs, eventually releasing miRISC complexes to possibly elicit further rounds of repression, which is represented by a relative increase in the mobility of miRNA particles in living cells and an increase in the population of monomeric miRNAs in fixed cells (Fig 4).”

Page 11:

“Time dependent changes in diffusion and assembly were observed in cells microinjected with let-7-a1 miRNA, supporting the model in Fig 4, featuring two kinetically distinct processes wherein miRNAs first assemble into large RNPs (such as a miRISC bound to an mRNA or PB), then are released from these complexes.”

Also, it is unclear if the "changes" in Fig. 3E and Supplementary Fig. S9 are really statistically significant.

Addressing the reviewer's question about the "changes" in Fig 3E and Supplementary Fig S9 (now Supplementary Fig S6), they are statistically significant with greater than 95% confidence based on a paired t-test ($p = 0.034$) between the 4 h and 32 h time points. This fact is now specified in the figure legend of Fig 3. Additionally, the data were fit best with a double-exponential function, signifying two kinetic processes.

Moreover, if the proposed model (based on Fig. 3E) is true, then the "fast" complex ($0.26 \mu\text{m}^2/\text{s}$; RNP or target-bound RISC) should increase at later time points in Fig. 2G, but it is apparently not the case.

The reviewer is correct, but his/her keen observation of a lack of an increase in 'fast' fraction is caused by limitations inherent to single molecule observation. As the residence time of microinjected miRNAs within cells increases, both 'slow' and 'fast' complexes indeed become steadily faster. However, our ability to detect particles with mobility greater than the 'fast' complexes in live cells is strongly impaired by our limited time resolution (~ 100 ms), thus resulting in an apparent decrease of the 'fast' fraction. To clarify this issue, we have modified **Fig 2F, 2G** and **Supplementary Fig 1E** to include a shaded region that reflects particles with even larger diffusion coefficients that are inaccessible at our 100 ms time resolution. We have also included the following statement in the main text:

Page 8:

"We note that the shifted distributions measured at later time points became increasingly curtailed above a diffusion coefficient of $\sim 1 \mu\text{m}^2/\text{s}$ (Fig 2F,G and Supplementary Fig S1E) due to our limited time resolution, somewhat diminishing the observed effect by narrowing the fast diffusing population."

Minor points:

1. *The first line in introduction: "RNA interference (RNAi)" should read "RNA silencing". These two words used to be used as very similar meanings, but RNAi now specifically refers to phenomena that accompany cleavage of complementary targets.*

As suggested, we have now changed this line in the introduction to read "RNA **silencing**".

2. *The labels and legend of Fig 1 are very confusing. What do "-ve" and "Ambion-vectrl" stand for? What's the difference between the second and third samples? Probably the third sample was with the mut target plasmid, not wt*

We have modified **Fig 1** to improve clarity (see also point 2 above).

3. *Supplementary Fig. S9 should show the assayed times (20 min to 32 h) as in Fig. 2G. Also, 32 h is longer than the cell division time and should thereby be avoided.*

We have modified this figure (now **Supplementary Fig 6**) to show the assayed times as suggested. We assayed cells after 32 h to ensure that microinjected cells divided normally and still contained fluorophore labeled miRNAs, thus attesting to the low invasiveness of our method and the high stability of fluorophore tagged miRNAs, respectively. The number of fluorescent particles at earlier time points was ~ 2 -4 fold higher than that present at 32 h, as expected after cell division.

Referee #3: This referee positively notes that the "paper is technically solid and provides important data for constructing mechanistic models of miRNA function." S/he appreciates the elegance of our method by stating that the "method appears relatively accessible to other researchers as well, which I think increases the potential impact of the work." S/he additionally goes on to commend our effort by stating "Most of the controls seem relevant and informative, and their approach seems to be sound.... In summary, I think this is a solid piece of work that could make a real contribution to the miRNA field, as well as linking that field to the biophysics field", but s/he also expresses a few concerns, addressed in the following.

1. *My one major issue is one of interpretation. The authors observe two populations of spots: one with a diffusion coefficient of around $.26 \mu\text{m}^2/\text{s}$ and one with a diffusion coefficient of around $0.034 \mu\text{m}^2/\text{s}$. They claim that the first population is most likely miRNA/target RNP complexes, whereas the second population corresponds to miRNAs in P-bodies. As far as I can tell, in their view, the hierarchy is:*

miRNA: too fast to detect
miRNA-AGO: too fast to detect
miRNA-AGO-RNP: $D=0.26$
miRNA-AGO-RNP-P-body: $D = 0.034$

Based on other literature from other groups (Golding et al. PNAS 2004, Shav-Tal et al. Science 2004, Vargas et al. PNAS 2005), it seems that the most likely that mRNP diffusion coefficients are closer to the 0.034 value than 0.26. Moreover, the nature and dynamics of P-bodies is somewhat mysterious and certainly the case for miRNA/P-body associations is a relatively new one that has not yet been fully characterized. One alternative hierarchy could then be:

miRNA: too fast to detect
miRNA-AGO: $D=0.26$
miRNA-AGP-RNP: $D=0.034$
miRNA-AGO-RNP-P-body: undetectable (for other reasons)

There is considerable variation in the diffusion constants reported in the literature for these complexes. The mRNAs tracked in Shav-Tal et al. Science 2004, Golding et al. PNAS 2004 and Vargas et al. PNAS 2005 were labeled such that each molecule theoretically bound ~48, 96 or 180 fluorescent MCP-GFP fusion proteins. Such constructs naturally display slowed mobility and small diffusion coefficients, on the order of $0.034 \mu\text{m}^2/\text{s}$, owing to a probe-induced increase in molecular weight. In contrast, we are probing endogenous mRNAs that do not contain such high molecular weight appendages and thus may diffuse faster than previously reported mRNA reporter constructs. Previous reports (Politz et al., Mol. Biol. Cell, 2006, Vol. 17, p1239-1249; Tadakuma et al., BBRC, 2006, Vol. 344, p772-779) that probed the diffusion coefficients of native mRNAs and those labeled with very few (< 8) fluorescent probes per mRNA found diffusion coefficients of $\sim 0.2\text{-}0.6 \mu\text{m}^2/\text{s}$, a range very much consistent with our measurements ($0.26 \mu\text{m}^2/\text{s}$). Moreover, we measure microscopic diffusion coefficients, computed over short time spans (300 ms), that may be higher than macroscopic diffusion coefficients as reported by Shav-Tal et al. Science 2004, Golding et al. PNAS 2004 and Vargas et al. PNAS 2005, which were computed over long time spans ($> \sim 1$ s), sometimes even over the entire visualization span of a particle. Such a systematic difference is explained for mRNAs that move rapidly over a short time range, but tend to occasionally get localized over a longer time range (Tadakuma et al., BBRC, 2006, Vol. 344, p772-779; Ishihama et al., BBRC, 2009, Vol. 381, p33-38).

I think the authors need to perform some experiments in order to distinguish between these possibilities. For instance, a co-stain with well-known P-body markers (either fused to GFP in live cells or IF in fixed cells) would be sufficient to show that the fraction they claim to be in P-bodies are actually in P-bodies (I realize that Pillai 2005 did some sort of demonstration of this, but I think an independent assessment would be wise considering that these labeled miRNAs may behave differently). Perhaps an AGO/RISC GFP fusion might also shed some light on the nature of the fast-moving particles the authors observe. Another thing the authors could do is perform single molecule FISH on the target after fixation to see what fraction of any particular target is bound to miRNA.

We now have added new data (**Supplementary Fig 2**) showing the colocalization of microinjected miRNAs with cognate mRNA targets and processing bodies (PBs), see also referee 2, point 3 above. We find that a significant fraction of Cy5 labeled miRNAs co-localize with both MS2-GFP labeled target mRNAs and GFP tagged Dcpl1a punctae, a principal component of PBs. Finally, the mobility of free Ago proteins and those bound to miRNAs is much higher than that of our miRNA particles (Ohr et al., Nucleic Acids Res., Vol. 36, p6439-6449), effectively blurring out at our ~ 100 ms time resolution. To clarify this issue we have added the following to the main text:

Page 7:

“Analysis of a large number of trajectories (**Supplementary Table 1**) 4 h after microinjection revealed two distinct Gaussian distributions of **microscopic** diffusion constants, with average diffusion coefficients of $\sim 0.26 \mu\text{m}^2/\text{s}$ and $\sim 0.034 \mu\text{m}^2/\text{s}$ for both let-7-a1-Cy3 (**Fig 2F**) and let-

7-a1-Cy5 miRNA (**Fig 2G**), smaller than those reported for RISC by fluorescence correlation spectroscopy, $\sim 5.4 \mu\text{m}^2/\text{s}$ (Ohrt *et al*, 2008). We did not observe more rapidly diffusing particles possibly due to limits in our time resolution (100 ms camera integration time) and/or since they may not be visible for all of 9 frames, our threshold for diffusion coefficient calculation. The main particles we did observe had diffusion coefficients that resemble those of messenger RNPs (Fusco *et al*, 2003) and PBs (Aizer *et al*, 2008), key mechanistic intermediates of mRNA repression by miRNAs (Bartel, 2009; Czech and Hannon, 2011) that have much higher molecular masses than RISC. These assignments were further supported by the observed colocalization of microinjected miRNAs both with mRNPs containing matching miRNA target sites and with PBs (**Supplementary Fig S2**). Taken together, our microinjected miRNAs are functionally fully active (**Fig 1**), and are associated with mRNAs and PBs (**Supplementary Fig S2**), strongly suggesting that the observed particles must contain miRNAs bound to and actively repressing target mRNAs.”

2. *The authors might argue that they do see a population that requires multiple steps to photobleach, which perhaps they would assume to then be P-bodies. However, I'm assuming many of the endogenous targets for these particular miRNAs in single cells have multiple miRNA binding sites per mRNA, in which case, one might see multiple photobleaching steps for labeled miRNAs when they are bound to the target mRNP even if they are not in P-bodies. I think the authors should certainly at least examine the number of potential targets for which there are multiple miRNA binding sites.*

We appreciate the authors concern; in fact, we initially expected this to be the case. However, based on the newly added experiment (**Supplementary Fig 7**), wherein we co-microinjected a target mRNA bearing six binding sites for an artificial cxcr4 miRNA, we believe that a majority of mRNA targets predominantly binds miRNAs at sub-stoichiometric ratios. This notion is based on the observation that introduction of the mRNA induces an increase in the number of multimers in our experiment, but not an enrichment of particles bearing six fluorescent probes.

Other, relatively less major issues:

1. *The authors say that cells fixed after just 20 minutes have high levels of background. This would imply that the number of labeled miRNAs decreases over time, suggesting that there is either some degradation (and subsequent ejection of the dye) or that the cell somehow expels the labeled miRNAs. In either case, this means that the authors' estimate of the number of miRNAs they microinject may not be accurate. Can the authors comment on this point, and more generally, on the degradation/export properties of their probes?*

We believe that the background mainly originates from free microinjected miRNAs before they assemble into larger RNPs. Considering that the 3' end of the miRNA is blocked against poly-uridylation by the fluorophore and microinjected cells contain fluorophore tagged miRNAs still 32 h after injection (**Supplementary Fig 6**), degradation of the miRNA is expected and appears to be relatively limited. We additionally find that the number of miRNA particles is very similar for most time points assayed (**Supplementary Table**), contrary to the notion that cells significantly expel labeled miRNAs over the time course of our experiments. We do observe, however, a decrease in the number of miRNA particles at very long incubation times (32 h), which may simply represent dilution of microinjected miRNAs upon cell division. Still, miRNA degradation, cleavage of the fluorophore from the miRNA, and/or efflux of labeled miRNAs from the cell cannot be completely ruled out. To clarify this issue we have added the following to the main text:

Pages 7/8:

“Notably, after 32 h the 3' fluorophore labeled miRNAs, whose 3' ends are blocked against poly-uridylation as a first step in the miRNA degradation pathway (Yeom *et al*, 2011), remained detectable as intracellular particles of relatively slow diffusion, suggesting that they were neither (fully) degraded nor exported.”

Page 9:

“We also note that the number of miRNAs we observed in the focal plane of a microinjected cell (~ 500 on average per fixed cell, considering both the number of distinct particles and their

individual photobleaching steps; **Supplementary Table 1** and **Fig 3D**) correlates well with the volume fraction of the cell in focus multiplied with the total number of microinjected miRNAs (~18,000), suggesting that we can account for a significant fraction of the microinjected miRNAs.”

2. *The figures are rather confusing to parse, with few indicative labels on the graphs. I suggest considering ways to make the figures easier to understand. For example: -Figure 1a has a variety of graphs labeled with things like -ve*, WT*, etc., that are perhaps understandable to those in the miRNA field, but are somewhat confusing to those outside it. Perhaps a diagram?*

As suggested by both referees 2 (see above) and 3, we have modified the figure in question (**Fig 1a**) to avoid confusion and better conform to conventions in the field.

3. *Figure 1b is very hard to interpret without delving into the legends and text.*

We have similarly modified **Fig 1B,C**.

4. *Figure 3E has no indication on what the dotted lines and solid lines represent. Again, these are merely stylistic suggestions-nothing in the graphs is misleading in any way.*

We initially had this information only in the figure legend, but have now added it directly to the figure (**Fig 3E**).

In summary, we here have carefully combined microinjection, optimized illumination strategy, single particle tracking (SPT) and the stochastic stepwise photobleaching of single fluorophores into one widely usable single-molecule tool. We further directly monitor here the intracellular diffusion and distribution of miRNAs and begin to unravel the complex nature of the biological processes involved in RNA silencing. We sincerely believe that we have fully addressed all concerns of the reviewers, in the process making our manuscript worthy of publication in *EMBO reports*.

None of the material has been published or is under consideration for publication elsewhere, including the internet. Thank you very much for your continued consideration.

2nd Editorial Decision

27 February 2012

Thank you for the submission of your revised study to EMBO reports. We have now received the assessment of the three referees who also saw your initial submission. As you will see, although referee 3 is now supportive of the study, referees 1 and 2 still have serious concerns that preclude acceptance of the work in its present form.

As both are very specific in their remaining concerns, I have decided to offer you an exceptional second round of revision. I appreciate that the concerns, especially those of referee 2, are not trivial to address. However, please note that EMBO reports would only be prepared to accept your study if you do address them in full. I would therefore understand if you rather chose to seek publication elsewhere at this stage. In addition, as supplementary figure 2 illustrates a crucial point of this study, it should be included in the main text, once appropriately controlled.

I look forward to seeing a revised form of your manuscript when it is ready. If you choose to submit the article elsewhere, please let us know.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The revised manuscript by Pitchiaya, et al, has been significantly improved compared to the previous version. The changes made as suggested by all three reviewers are satisfactory, and the new experiments and data added enhanced the conclusions of the manuscript significantly. However, there are still few places need to be further revised. I would recommend the publication of this manuscript as long as my additional comments can be addressed.

1. Regarding my previous concern on the counting of photobleaching steps for the exogenous labeled miRNAs and their competition against endogenous miRNAs for binding to the target mRNAs, I agree with the authors' explanation that "the number of microinjected let-7 miRNAs is similar to that already present endogeneously, hence one would expect about equal probability to encounter a labeled and unlabeled let-7 miRNA molecule in any given complex", and that most of their conclusions are drawn depending on "the changes over time of the relative (not absolute) numbers of a given miRNA assembled into RNPs and PBs". Therefore, I am happy about the changes made in the revised manuscript. However, I think sentence such as "stepwise photobleaching reveals that the largest fraction of these particles contains single miRNA molecules..." (in the last paragraph for summary) should also be revised accordingly, since this sentence seems to generalize the conclusion to all miRNAs including the undetectable unlabeled ones.

2. The new experiments on the colocalization of miRNA with one of PB components and target mRNA provide strong evidence for the functional relevance of the targeted and PB-binding miRNA inferred from the diffusion analysis. However, it would be even stronger if the authors can selectively track the miRNA particles that associated with PB component, and show that the diffusion coefficient falls into the range of the one proposed for PB-binding miRNA particles.

3. In response to my previous comment regarding the lack of time-dependent change in the relative population of single and multiple miRNAs contained particles for cxcr4 miRNA, in the revised manuscript, the authors have performed new experiments to support their interpretation that this is due to the fact that cxcr4 has 10-fold few mRNA targets and therefore the change is too small to be detected. In these experiments, target mRNAs were microinjected together with the cxcr4 miRNAs and they observed an increase in the fraction of multiple miRNAs contained particles compared to control experiments. However, it is not entirely satisfactory that the authors did not further show a time-dependent curve for the fraction change as the one for let-7-a1 miRNA, therefore it cannot be considered as direct evidence to support their original interpretation.

4. Some minor points:

- 1) In supplementary methods on live cell imaging data analysis, supplementary Fig 2 is mis-referenced at the bottom of page 16. The correct figure for this part should be Supplementary Fig 1. In addition, Supplementary videos 1A and 1B are not referenced in the correct places in the legend of Fig. 2. Based on the supplementary information, video 1A and 1B are for let-7-a1-cy3 and let-7-a1-cy5, respectively, instead of for let-1-a1-cy5 at 2h and 4h post microinjection.
- 2) The description of Fig. 1B in the text somehow does not read very clear. On page 5, "we microinjected either mutant or wild-type let-7-a1 along with a mutant target and control fluorescent reporter gene plasmid and..." is better revised as "... neither mutant or wild-type let-7-a1 along with a mcherry reporter plasmid containing a mutant target and a control GFP reporter plasmid..."

Referee #2:

The authors have addressed a number of my previous concerns, merely by rewriting the text rather than performing additional control experiments. However, the most important control-validation of target-bound RISC-is still missing. Newly added Supplementary Fig. 2 lacks critical controls and does not address any target specificity of these loci. The authors must perform a series of control experiments to validate that such co-localization is dependent on sequence-specific target binding of RISC (e.g., by using Cy5-labeled unrelated small RNA duplexes, non-specific mRNA reporters, and/or specific/non-specific 2'-O-Me inhibitors etc.) and quantify and compare the co-localizing frequencies. Moreover, the authors should show that the numbers of photobleaching steps are significantly different between co-localize (target-bound) foci and independent (target-free) foci.

Minor point:

According to the authors' description, iSHiRLoC is simply a combination of previously established methods, and I'm not sure if its methodological novelty is sufficient for giving a new, fancy name.

Referee #3:

The authors have addressed my concerns and I think the paper is suitable for publication.

2nd Revision - authors' response

28 April 2012

In the following, please find our point-by-point responses to the reviewers' comments and suggestions.

Referee#1:

1. Regarding my previous concern on the counting of photobleaching steps for the exogenous labeled miRNAs and their competition against endogenous miRNAs for binding to the target mRNAs, I agree with the authors' explanation that "the number of microinjected let-7 miRNAs is similar to that already present endogeneously, hence one would expect about equal probability to encounter a labeled and unlabeled let-7 miRNA molecule in any given complex", and that most of their conclusions are drawn depending on "the changes over time of the relative (not absolute) numbers of a given miRNA assembled into RNPs and PBs". Therefore, I am happy about the changes made in the revised manuscript. However, I think sentence such as "stepwise photobleaching reveals that the largest fraction of these particles contains single miRNA molecules..." (in the last paragraph for summary) should also be revised accordingly, since this sentence seems to generalize the conclusion to all miRNAs including the undetectable unlabeled ones.

As suggested by the referee, we have now also modified the abovementioned statement such that it is specific to microinjected miRNAs, as follows—

Page 11: "Stepwise photobleaching revealed that the largest fraction of these particles contains single **fluorophore labeled** miRNA molecules"

2. The new experiments on the colocalization of miRNA with one of PB components and target mRNA provide strong evidence for the functional relevance of the targeted and PB-binding miRNA inferred from the diffusion analysis. However, it would be even stronger if the authors can selectively track the miRNA particles that associated with PB component, and show that the diffusion coefficient falls into the range of the one proposed for PB-binding miRNA particles.

We appreciate the referee's comments and agree that it would be ideal to co-track miRNAs with PBs; in fact, we are planning such experiments in the future. However, such multi-

color tracking mandates extensive modifications to our current optical setup and is thus beyond the scope of the current report. Experimentally practically equivalent, however, we have now tracked single PBs in live HeLa cells and directly show in **Supplementary Fig. 2** that these punctate structures bear diffusion coefficients very similar to those of PB-binding miRNA particles, i.e. the slowly diffusing fraction of miRNAs (**Fig. 2F** and **G**) in our live cell tracking experiments.

3. In response to my previous comment regarding the lack of time-dependent change in the relative population of single and multiple miRNAs contained particles for cxcr4 miRNA, in the revised manuscript, the authors have performed new experiments to support their interpretation that this is due to the fact that cxcr4 has 10-fold few mRNA targets and therefore the change is too small to be detected. In these experiments, target mRNAs were microinjected together with the cxcr4 miRNAs and they observed an increase in the fraction of multiple miRNAs contained particles compared to control experiments. However, it is not entirely satisfactory that the authors did not further show a time-dependent curve for the fraction change as the one for let-7-a1 miRNA, therefore it cannot be considered as direct evidence to support their original interpretation.

We have now added the suggested data to **Fig. 4E**, depicting the time dependent changes in cxcr4 miRNA assembly in new experiments where specific mRNA targets bearing six cxcr4 seed sequences were co-microinjected along with the miRNA. The data clearly show again two kinetically distinct assembly pathways (with rate constants of $1.14 \pm 0.12 \text{ h}^{-1}$ for the faster phase and $0.30 \pm 0.07 \text{ h}^{-1}$ for the slower phase), similar to our observations with let-7-a1 (rate constants of $1.2 \pm 0.2 \text{ h}^{-1}$ for the faster phase and $0.14 \pm 0.08 \text{ h}^{-1}$ for the slower phase). These results clearly validate the absence of temporal changes when no target mRNA is present and fully support the notion that intracellular miRNA assembly is dependent on the abundance of specific mRNA targets. Consequently, we have made the following changes to the main text—

Page 10: “**By contrast, when cxcr4 was co-microinjected with ~3,000 specific mRNA target molecules, miRNA assembly adopted again two kinetically distinct phases (with rate constants of $1.14 \pm 0.12 \text{ h}^{-1}$ and $0.30 \pm 0.07 \text{ h}^{-1}$; Fig 4E and Supplementary Fig S7A,B).**”

4. Some minor points: 1) In supplementary methods on live cell imaging data analysis, supplementary Fig 2 is mis-referenced at the bottom of page 16. The correct figure for this part should be Supplementary Fig 1. In addition, Supplementary videos 1A and 1B are not referenced in the correct places in the legend of Fig. 2. Based on the supplementary information, video 1A and 1B are for let-7-a1-cy3 and let-7-a1-cy5, respectively, instead of for let-1-a1-cy5 at 2h and 4h post microinjection. 2) The description of Fig. 1B in the text somehow does not read very clear. On page 5, "we microinjected either mutant or wild-type let-7-a1 along with a mutant target and control fluorescent reporter gene plasmid and..." is better revised as "... neither mutant or wild-type let-7-a1 along with a mcherry reporter plasmid containing a mutant target and a control GFP reporter plasmid..."

We thank the referee for his/her careful reading to find these minor errors. As suggested, we now have:

- a. correctly referenced **Supplementary Fig S1A** on Page 23 (formerly 16) of the supplementary information
- b. modified the legend of Fig. 2 on page 16 such that the Supplementary Videos are now correctly referenced, as follows—
 “**Pseudo-colored images of cells microinjected with (A) let-7-a1-Cy3, (left, cytoplasmic injection; right, nuclear injection, Supplementary Video 1A) imaged 4 h after microinjection and (B) let-7-a1-Cy5 (Supplementary Video 1B) imaged 2 h after microinjection, showing distinct particles containing miRNAs.**”
- c. Modified the main text on page 5 such that the description of Fig. 1B is clearer, as follows—
 “**Finally, we microinjected either mutant or wild-type let-7-a1 along with both an mCherry reporter plasmid containing seven mutant let-7 target sequences and a control GFP reporter plasmid, and found that the intended target protein was specifically and strongly repressed, supporting the biological relevance of**

microinjecting miRNAs as part of iSHiRLoC (**Supplementary Methods and Fig 1B,C**).”

Referee#2:

The authors have addressed a number of my previous concerns, merely by rewriting the text rather than performing additional control experiments. However, the most important control-validation of target-bound RISC-is still missing. Newly added Supplementary Fig. 2 lacks critical controls and does not address any target specificity of these loci. The authors must perform a series of control experiments to validate that such co-localization is dependent on sequence-specific target binding of RISC (e.g., by using Cy5-labeled unrelated small RNA duplexes, non-specific mRNA reporters, and/or specific/non-specific 2'-O-Me inhibitors etc.) and quantify and compare the co-localizing frequencies. Moreover, the authors should show that the numbers of photobleaching steps are significantly different between co-localize (target-bound) foci and independent (target-free) foci.

To address the referee's concerns, we have now added new control experiments in the form of **Fig 3C** (moved from the Supplement to the main text for added weight) that clearly show the specificity of miRNA-target mRNA co-localizations. In short, we found that the co-localization frequency of specific (mutant let-7-a1) miRNAs with mRNAs containing mutant let-7-a1 target sequences are significantly higher than those of non-specific (wild-type let-7-a1) miRNA with the mutant target and other non-specific colocalizations. Additionally and as suggested by the referee, found that the distribution of photobleaching steps in such co-localized (target-bound) miRNA particles is significantly different from those in independent (non-co-localized) foci (new **Supplementary Fig S7C**), further supporting our model that miRNA assembly is mRNA dependent. Accordingly, we have made the following changes to the main text—

Page 10: “**Additionally, and as expected from our model, miRNAs shifted towards multimeric assemblies when we found them co-localized with target mRNA, but were more monomeric in the absence of a co-localized target (Supplementary Fig S7C). Taken together, we find strong evidence for mRNA dependent miRNA assembly.**”

Minor point: According to the authors' description, iSHiRLoC is simply a combination of previously established methods, and I'm not sure if its methodological novelty is sufficient for giving a new, fancy name.

We agree with the referee that iSHiRLoC combines several previously established methods, but in a novel way to study the transport, localization and assembly of miRNAs in their native environment, which has not been accomplished before. We have modified and shortened the segment describing these facts, as follows—

Page 3/4: “**The strategies available so far, however, either decorate the target molecule with multiple fluorescent probes, thereby creating high-molecular weight appendages that may impede function, and/or have only been applied to proteins or large RNA-protein complexes [9, 11, 12]. Our intracellular single molecule, high-resolution localization and counting (iSHiRLoC) method overcomes these caveats by combining microinjection (which defines an experimental start point) and low-background illumination with SPT and stepwise photobleaching to visualize the diffusive motions and distribution of singly fluorophore labeled functional small RNAs inside cells.**”

In addition, since we predominantly use the acronym to define our method in different parts of the text for the sake of brevity, we feel that it remains warranted.

In conclusion, we appreciate the constructive criticisms of the reviewers, which have helped make our manuscript stronger yet and, we believe, worthy of publication in *EMBO reports*. None of the material has been published or is under consideration elsewhere, including the Internet.

Thank you very much for your time and continued consideration.

Thank you for the submission of your revised manuscript and your patience while it has been peer-reviewed once again. Referee 1 was unavailable to look at the study and referee 2 has therefore assessed the answers to all remaining concerns. As you will see below, although s/he is now overall positive towards publication of the study, there are three minor issues that need to be taken care of before we can accept your study for publication.

Once these three points have been taken care of, we would be in a position to accept your study for publication. As I mentioned in my previous letter, it is EMBO reports policy to allow a maximum time of six months between initial and final decision. In this case, this means that we would need to receive the final version of your study by June 4th.

I look forward to seeing a final version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #2:

The authors have adequately addressed most of the previous concerns from Referee #1 and myself. I have some comments on the newly added Fig. 3C.

1. To draw error bars, 3 or more biological replicates are required (currently $n=2$).
2. The authors should show that the densities of red and green spots are comparable between "specific" and "non-specific" samples.
3. The authors used wild-type let-7-a1 sequence as the "non-specific" miRNA. However, because let-7-a1 is a natural miRNA, there should be many endogenous mRNAs targeted by let-7a-1, which might sequester the fluorescently labeled miRNAs away from binding to the reporter mRNA labeled with EGFP. Thus, the authors should use a completely different small RNA (such as *cxcr4*) and a reciprocal reporter mRNA (as in Fig. 4E) as controls.

Referee#2:

1. *To draw error bars, 3 or more biological replicates are required (currently $n=2$).*

We agree with the referee that three or more replicates are preferred (although only two are required) to draw error bars. The challenging nature of our experiments, however, leads to practical limitations, and the statistical variation within each sample group was minimal. To clarify the nature of our data, we have now modified **Fig 3C** and **Supplementary Fig S7C** to explicitly show each data point and thus the statistical variation (and resulting

mean) we observe in each replicate. In addition, we have added the following to the main and supplementary texts:

Main text, page 18: “Fraction of specific or non-specific miRNA co-localizing with mRNA-EGFP. Cells were injected with the MS2/MCP plasmids and either mutant (specific; **circle**) or wild-type (non-specific; **square**) let-7-a1. To test for the contribution of background fluorescence, control cells were injected with mutant let-7-a1 and MCP plasmid without the MS2-mRNA expression plasmid (specific; **triangle**). **Data points within each group and their associated means (dashed lines) are shown (n = 2 cells for each group).**”

Supplementary text, page 7: “**Data points within each group are explicitly shown (grey circles, monomers; black squares, multimers) in addition to their mean values (grey dotted line, monomers; black dotted line, multimers). The statistical variation within each group was minimal between replicates.**”

2. *The authors should show that the densities of red and green spots are comparable between "specific" and "non-specific" samples.*

As suggested, we now report on the density of the red and green spots for both the “specific” and “non-specific” samples, as follows:

Supplementary Methods, Page 26: “**We typically observed a density of 0.08 Cy5 particles/ μm^2 , 0.28 GFP particles/ μm^2 and 0.13 Cy5 particles/ μm^2 , 0.2 GFP particles/ μm^2 in the specific (in the presence of target mRNA) and non-specific (with a negative control miRNA) samples, respectively. The density of green particles arising from background fluorescence was 0.08 GFP particles/ μm^2 in samples that were injected with mutant let-7-a1-Cy5 (0.07 particles/ μm^2) and MCP plasmid without the MS2-mRNA expression plasmid.**”

These data show clearly that the densities in these samples are comparable and the co-localization frequency is not merely due to chance co-occurrence, but is dependent on mRNA/miRNA seed sequence pairing.

3. *The authors used wild-type let-7-a1 sequence as the "non-specific" miRNA. However, because let-7-a1 is a natural miRNA, there should be many endogenous mRNAs targeted by let-7a-1, which might sequester the fluorescently labeled miRNAs away from binding to the reporter mRNA labeled with EGFP. Thus, the authors should use a completely different small RNA (such as cxcr4) and a reciprocal reporter mRNA (as in Fig. 4E) as controls.*

We feel that let-7-a1 is in fact a very good “non-specific” miRNA control for the following reasons:

- a. Because let-7-a1 targets more endogenous mRNAs than an artificial miRNA such as cxcr4, we expect in fact a greater extent of sequence-independent co-localization from let-7-a1— not less as the reviewer suggests. The reason is that we have evidence that we observe two types of miRNA-mRNA co-localization events in our experiments: (1) binding of the miRNA directly to the mRNA and (2) chance co-occurrence of the miRNA and mRNA in the same cellular focus, such as a P-body, physically separated from one another at a distance less than the optical resolution limit. Since let-7-a1 has many more endogenous targets than cxcr4, as the reviewer notices, it represses these targets and thus localizes them to P-bodies, where it then has a higher probability of residing in the vicinity of a fluorophore labeled reporter mRNA that associates with the P-body due to the action of an unlabeled let-7 or other miRNA, or more simply due to normal cellular turnover. Following this logic, we expect a cxcr4 control RNA to show fewer “non-specific” co-localizations with the expressed mutant mRNA reporter and thus provide an artificially low “non-specific” background count.

- b. All our data have to be considered together. In particular, our luciferase reporter gene assays indicate that the wild-type let-7-a1 does not repress the reporter bearing mutant let-7-a1 seed sequences (Fig 1A), directly demonstrating that seed sequence pairing is a requirement for repression and potentially reflecting low engagement of the miRNA with the mRNA, which is further confirmed in our co-localization assays.

Consequently, we have added the following to the Supplementary Methods:

Page 26: “We chose wild-type let-7-a1 as a non-specific control miRNA for two reasons: (i) This miRNA is incapable of repressing a reporter target with mutant let-7-a1 seed sequences (Fig 1A) and (ii) we expect maximal non-specific co-localization of this miRNA with our reporter mRNA in common cellular RNA foci, such as PBs, since let-7-a1 has many endogenous targets that it would localize to PBs, where the mutant mRNA may also reside due to its regulation by endogenous factors. Fluorophore labeled wild-type let-7-a1 thus serves as a robust negative control miRNA, setting an upper limit for sequence independent co-localization.”

Based on these reasons and our finding that miRNA assembly is target mRNA dependent (Fig 4E), using a completely different small RNA and creating reciprocal reporter constructs we feel is not necessary and beyond the scope of the current manuscript.

In conclusion, we appreciate the repeated constructive criticisms of this reviewer, who has shown a remarkable tenacity and detail orientation. His/Her suggestions have helped make our manuscript clearer and, we believe, now finally worthy of publication in *EMBO reports*. None of the material has been published or is under consideration elsewhere, including the Internet.

Thank you very much for your time and continued consideration.

4th Editorial Decision

16 May 2012

Thank you for the submission of the revised version of your study to EMBO reports. I think it adequately addresses all remaining concerns and, thus, I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports