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RNA Localization: Visualization in Real-Time

Dispatch

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RNA localization during development is required for proper sorting of developmental determinants. Direct visualization of endogenous transcribed RNA is now possible and should provide new insights into how this process occurs.

One of the potential capabilities of recent advances in microscopic technology is to unify molecular, cellular and developmental biology by allowing gene expression to be visualized in living cells. The ultimate goal of this technology is to extend this detection to developing organisms. This goal has now been achieved by Forrest and Gavis [1], who reported recently in *Current Biology* that the localization of *nanos* mRNA can be visualized during development in living *Drosophila* egg cases.

Nanos is one of the small set of maternally expressed genes that pattern the posterior end of the developing fly embryo. The fly has an ovarian cyst consisting of one oocyte and 15 nurse cells, plus the somatic follicle cells. *Nanos* and other early patterning genes are expressed in the nurse cells, the RNAs being transported into the oocyte where they are localised in a specific manner relating to their patterning function [2].

To visualize RNA *in vivo*, Forrest and Gavis [1] used a method involving a fusion protein consisting of the green fluorescent protein (GFP) linked to the RNA binding coat protein of the phage MS2 [3]. The RNA of interest — *nanos* — was synthesized from a transgene into which binding sites for the fusion protein had been inserted. Using this approach, Forrest and Gavis [1] have gained insights into the process of RNA localization that could not have been obtained using earlier methodologies.

Prior to the work of Forrest and Gavis [1], two approaches were used to detect RNA localization in *Drosophila* oocytes. The earliest and most used method is *in situ* hybridization, predominantly with RNA probes labeled with digoxigenin [4]. The limitation of this technology is the signal-to-noise ratio, which determines the sensitivity. Because the probes have to penetrate into the oocyte in order to hybridize to the RNA, the larger the oocyte, the more the background and the less the penetration. The later stages in oocyte development, such as those occurring after nurse cell 'dumping' of cytoplasm into the oocyte, have thus been less tractable to this kind of analysis. The other limitation of *in situ* hybridization as an experimental approach is that the material is fixed and hence dynamic processes cannot be followed directly.

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This limitation was obviated by the injections of fluorescently labeled RNA, an approach pioneered by Ainger *et al.* [5] in oligodendrocytes and by Glotzer *et al.* [6] for *Drosophila*. The RNA can be tagged using fluorescent nucleotides during its *in vitro* transcription. The injection of such constructs into oocytes, and even into the cytoplasm of the syncytial blastoderm, has provided the best evidence to date that RNAs destined for localization to the periphery are associated with motor proteins, such as myosin, dynein or kinesin [3,7–10]. Live cell imaging has provided the ability to track these movements. The dynamics of this system allowed an initial, crude characterization of the motors involved in trafficking the RNA, by assessing their speed and directionality. The use of drugs that disrupt the cytoskeleton, antibodies against the proteins and RNA interference (RNAi) to downregulate expression all helped to identify specific motor proteins involved in this RNA localization. As endogenous RNA cannot be visualized using this method, the labeled non-functional RNA serves as a surrogate marker for the *bona fide* localization pathway.

While this approach may provide a good approximation for the various steps in the localization pathway, certain caveats should be remembered. The first is that the RNAs destined for localization may actually be recognized by the cellular machinery in the nucleus, where the RNA is first synthesized [11–13]. The pre-mRNA, or mature transcripts, may pick up a protein required, for instance, for translational repression or assembly with a motor. Naked RNAs micro-injected into the ooplasm form complexes with proteins [14], but these may not be identical to those forming under endogenous conditions. Second, RNAs may be structurally modified when they are fluorochrome tagged for visualization. Specific proteins that identify 'zipcodes' [15] in the RNA may not bind properly to such modified RNAs, possibly allowing binding by non-specific proteins [16]. A third problem is that microinjection of RNA will be unlikely to recapitulate either the timing or the level of its endogenous expression.

Another way of identifying RNAs is to use a GFP fusion to a known RNA-binding protein specific for localization [11,17]. Even if the fusion protein can be shown to bind the RNA specifically *in vitro*, however, it is at best an indirect indicator, as one is never sure that the fusion protein behaves the same *in vivo* and really binds the RNA of interest.

These experimental problems can be circumvented by the endogenous expression of the reporter RNA containing high-affinity binding sites for the phage protein MS2 fused to GFP [3]. Because it contains a nuclear localization signal, the fluorescent fusion protein remains in the nucleus until it binds to the RNA — presumably during transcription — and then is transported with the RNA out of the nucleus. Therefore, the fluorescent signal in the cytoplasm results only from the association with RNA. This system was

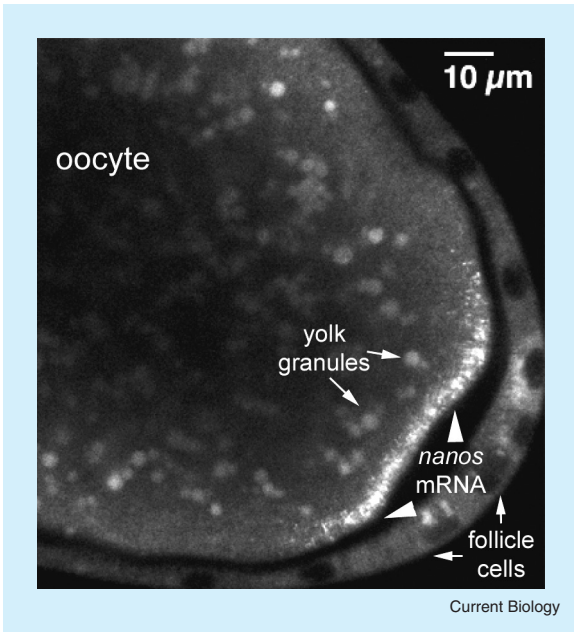

Figure 1. Localization of *nanos* RNA in the living *Drosophila* egg chamber.

The bright particles of mRNA (arrowheads) can be seen accumulating at the posterior cortex at stage 10b of oogenesis. The yolk granules and follicle cells (arrows) can be easily distinguished from the RNA.

used initially in yeast [3,18] and then in cultured mammalian cells [19,20], but previously had not been used in tissues or entire organisms.

Forrest and Gavis [1] labeled *nanos* RNA using a two-part reporter system of this kind (Figure 1). The modified *nanos* RNA behaves the same as the normal endogenous RNA, at least in the localization assay used. Importantly, the RNA reporter rescues a *nanos* deletion mutant. The authors graphically demonstrate the movement of the RNA in movies taken during oogenesis, which reveal features of the RNA movement heretofore unseen. Because this approach labels the RNA without being invasive, and thus allows the RNA to follow its native pathways, this methodology facilitates the study of the kinetics of *nanos* movements, as well as those of other RNAs. It will provide a means to critically dissect each component of the pathway, spatially and temporally. Some benefits of this approach are already evident in the work reported in the Forrest and Gavis [1] paper.

Some significant observations result from the ability to visualize the RNA *in vivo*. One is that, despite the interference from the yolk granules, the system appears to be more sensitive than *in situ* hybridization, particularly at the later stages of oogenesis. This means that lower levels of RNA can be detected. For instance, this has allowed Forrest and Gavis [1] to see when *nanos* RNA is first localized, and this turns out to be much earlier than previously thought. The sensitivity of the system showed that, although a microtubule inhibitor suppresses cytoplasmic streaming, while decreasing significantly the amount of localized RNA, it does not eliminate it completely. Data of this kind provide mechanistic insights into the localization by allowing a quantitative assessment of precisely how much RNA localization is actually dependent on microtubules or cytoplasmic streaming, relative to diffusion. This clarifies previous work where *oskar* RNA localization was not dependent on microtubules if injected close to the posterior pole of the oocyte [6].

A similar approach differentiates the nature of the anchoring at the posterior cortex: low levels of actin disrupting agents prevent RNA localization, but not apparently anchoring. This, once again, demonstrates the precision of the experiment for both the analysis of timing and quality of the localization. Most interesting, however, is the loss of the anchoring point of the localized RNA aggregate when higher doses of actin disrupting agents are used. The supplementary movies demonstrate the loss of the RNA, not as individual molecules diffusing away, but as a large clump of germ plasm being swept away, much as a boat that has lost its mooring. This is another example of how this technology contributes insights into the localization mechanism. In particular, the approach allows visualization of the exact moment the detachment occurs, unlike previous approaches using fixed materials where one can only surmise what has happened prior to fixation.

Evidence presented at recent meetings suggests that endogenous RNA can behave differently from injected, labeled RNA. There may be many reasons for this. The important point, however, is that this work overcomes these concerns and will be an important contribution to the field of RNA localization in *Drosophila*. It may become the preferred approach for studying RNA transport and localization, refining and perhaps overriding interpretations from previous work that has depended on more invasive methods. Most likely it will be a complementary approach; microinjection makes it possible to 'place' a transcript in a particular site and to test the functionality of specific RNA–protein complexes. For instance, nurse cell cytoplasm, when mixed with microinjected RNA, was found to contain a factor needed for directional movement in the oocyte [10]. Both types of approach are now part of the toolkit provided for the investigator of RNA localization in living cells.

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