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Fixed and live visualization of RNAs in *Drosophila* oocytes and embryos

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ABSTRACT

The ability to visualize RNA *in situ* is essential to dissect mechanisms for the temporal and spatial regulation of gene expression that drives development. Although considerable attention has been focused on transcriptional control, studies in model organisms like *Drosophila* have highlighted the importance of post-transcriptional mechanisms - most notably intracellular mRNA localization - in the formation and patterning of the body axes, specification of cell fates, and polarized cell functions. Our understanding of both types of regulation has been greatly advanced by technological innovations that enable a combination of highly quantitative and dynamic analysis of RNA. This review presents two methods, single molecule fluorescence *in situ* hybridization for high resolution quantitative RNA detection in fixed *Drosophila* oocytes and embryos and genetically encoded fluorescent RNA labeling for detection in live cells.

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1. Introduction

The visualization of mRNA expression patterns is fundamental to deciphering the regulation and function of genes that control animal development. With its wealth of genetic and molecular tools, as well as tissue accessibility, *Drosophila* has long served as a model system for elucidating the temporal and spatial patterns of gene transcription that give rise to the segmental body plan. Studies in *Drosophila* have also led the way toward understanding the importance of intracellular mRNA localization in generating cellular and developmental asymmetry. Much work has focused on the analysis of maternal transcripts, whose localization in the oocyte and/or early embryo are essential for the establishment and patterning of the body axes and the specification and development of the germline [1,2]. In addition, a variety of localization patterns and functions for RNA localization in differentiated cells are coming to light, highlighting the versatility of this post-transcriptional regulatory mechanism [3]. Notably, large scale fluorescence *in situ* hybridization screens found that 71% of 3000 transcripts analyzed in the *Drosophila* embryo, and 22% of nearly 6000 analyzed in the ovary, are subcellularly localized [4,5].

Following their synthesis, localized mRNAs must be directed to the appropriate machinery for delivery to the correct region of the cell. As a general paradigm, RNAs are transported as ribonucleoprotein particles (RNPs); in most known examples, the RNPs attach to molecular motors for directed, cytoskeletal-based transport but in some cases they move by diffusion and become locally entrapped [1,6]. RNPs are built through the interactions of both transcript-specific and more general factors with sequence elements or structural motifs in the transcript, and the particular set of RNP components is thought to determine RNP behavior. They may also be remodeled or augmented for different stages of the localization process, for example through the recruitment of adaptors to motors for transport or proteins involved in anchoring at the target destination [3,7]. The mechanisms governing the formation of these RNPs, their specific RNA and protein content, and their dynamic behavior over the life of a transcript are areas of active investigation.

Our mechanistic understanding of mRNA localization has advanced as methods to detect RNA *in situ* have improved and expanded. The earliest *in situ* hybridization experiments to analyze RNA distributions in *Drosophila* oocytes and embryos were performed using radiolabeled probes applied to tissue sections [8,9]. Indirect detection of probes containing digoxigenin or biotin-conjugated nucleotides by enzyme-based immunohistochemistry, which greatly increased efficacy and sensitivity and could be applied to whole mount embryo preparations, soon became the method of choice [10]. These methods provided basic information about the location of a particular transcript within a cell as well as the first insights into RNA localization mechanisms, revealing the effects of genetic or pharmacological perturbations on the RNA distribution [11–15]. The advent of fluorescence *in situ* hybridization (FISH) improved spatial resolution and facilitated multiplex RNA detection (for a comprehensive review of FISH, see Levsky and Singer [16]). Further adaptations allowed FISH to be combined with immunofluorescence, permitting co-detection of RNA and protein. However, neither enzyme-based immunohistochemical detection nor detection by typical FISH probes synthesized with stochastically incorporated fluorophores allow for absolute RNA molecule quantification. The development of highly sensitive FISH methods capable of detecting single RNA molecules – single molecule FISH (smFISH) – now make it possible to quantify gene expression *in situ*. For the field of mRNA localization, the ability to detect transcripts quantitatively and to map their positions with high resolution by smFISH has opened the door to determining the precise molecular contents and assembly mechanisms of RNPs that mediate various stages in the life of an mRNA and its travels within a cell.

In situ hybridization is limited to a static view of the cell at a particular time, leaving the events that produce the final observed RNA distribution to conjecture. A full understanding of dynamic processes like mRNA localization requires the ability to visualize RNA molecules in live cells, in real time. Numerous methods have been developed to this end, including injection of *in vitro* synthesized fluorescently labeled transcripts and the application of conditionally fluorescent RNA-binding probes like molecular beacons, RNA aptamers, and RNA intercalating dyes (see Gaspar and Ephrussi for detailed review [17]). While these reagents can be readily delivered to cultured cells, introducing them into *Drosophila* oocytes and embryos is problematic, requiring microinjection or inefficient and potentially harmful permeabilization schemes. In contrast, genetically encoded fluorescent tagging methods based on the high affinity interaction of bacteriophage proteins with cognate RNA stem-loops [17] are particularly well suited for *Drosophila* given the ease of transgenesis.

Here we describe the application of smFISH and genetically encoded RNA tagging to the analysis of intracellular mRNA localization in *Drosophila* oocytes and embryo. Both of these methods can be combined with protein detection methods to determine the spatial relationships of RNA and protein. We also briefly discuss their use for measuring transcription and transcriptional dynamics. While we focus here on transcript visualization in oocytes and early embryos, both smFISH and *in vivo* RNA tagging are amenable to use in differentiated tissues at later stages of development.

2. Methods for detection of RNA in fixed oocytes and embryos by smFISH

The smFISH technique developed by Raj *et al.* [18] allows highly sensitive, quantitative RNA detection and can be easily multiplexed to monitor several RNA species simultaneously. In contrast to traditional FISH methods, which use one or several probes complementary to the target RNA that are generally hundreds of nucleotides in length, smFISH uses many short oligonucleotide probes arrayed along the target RNA (Fig. 1A). Each probe is coupled to a fluorophore and as a result, binding of the set of probes to the RNA produces a high-intensity point source that is detected as a diffraction limited spot; the sensitivity achieved by the high density of labeled probes affords single molecule detection. Moreover, because signal detection requires binding of a substantial number of probes, background due to non-specific probe binding is minimized; i.e., there is a high signal to noise ratio. These attributes allow accurate counting of RNA molecules within a cell. Importantly, the method is readily adaptable to many tissue and cell types [18]. The small size of the oligonucleotide probes has a particular advantage for the *Drosophila* ovary by allowing efficient penetration of late-stage oocytes, which are largely inaccessible to traditional probes [19,20] (Fig. 2). The protocol described here is adapted from Raj and Tyagi [21], and has been optimized for detection of transcripts in *Drosophila* ovaries and embryos by S. Little [19,22]. We refer the reader to the original protocol for the detailed rationale behind the procedures [21].

2.1. Probe design and preparation

Sets of oligonucleotide probes targeting an RNA sequence can be designed using a free web-based program, Stellaris FISH Probe Designer, developed by Raj *et al.* [18] and available at http://www.biosearchtech.com/stellarisdesigner/. The program optimizes GC content while allowing for customization of probe length