The molecular cytology of gene expression: fluorescent RNA as both a stain and tracer in vivo

T. Pederson
Program in Cell Dynamics and Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worchester, MA, USA

For more than 60 years, RNA has been detectable in fixed cells and tissues by relatively specific staining methods. More recently, it has become possible to study RNA in unfixed, live cells. This review article describes how the intracellular dynamics and localization of RNA *in vivo* can be studied by microinjection of fluorescent RNA into cells- an approach we have termed Fluorescent RNA Cytochemistry. Depending on the particular RNA species under investigation, Fluorescent RNA Cytochemistry can operate as a "stain" to reveal intracellular sites at which a given RNA resides, or as a "tracer" to allow movements of a dynamically translocating RNA to be followed in the living cell. Several examples of Fluorescent RNA Cytochemistry are presented, collectively illustrating the range of applicability this approach offers in the toolbox of gene expression, studied as *in vivo* cell biology.

Key words: real-time imaging, nucleolus, Cajal bodies, nuclear import and traffic of RNA.

Correspondence: Thoru Pederson, Program in Cell Dynamics and Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 377 Plantation Street, Worcester, MA 01605 USA.
Phone: international +1.508.8568667.
Fax: international +1.508.8568668.
E-mail: thoru.pederson@umassmed.edu

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When first working on nucleic acid colorimetric methods and cytochemistry as a graduate student (Pederson, 1969; Pederson and Gelfant, 1970), I was both impressed and intrigued that DNA and RNA could be observed at all in fixed cells. Unlike many histochemical reactions I had studied in class, where the target entity seemed highly available (like the liver's glycogen for the PAS reaction, or the hyalauronic acid/elastase components of connective tissue which seemed to me just waiting to be stained), my naïve student thinking envisioned nucleic acids to be so packaged and buried by proteins as to be non-ideal cytochemical targets. The detection of RNA in cells had been pioneered by Torbjorn Caspersson using not dyes but UV absorption cytophotometry in conjunction with the use of ribonuclease digestion, as introduced by Brachet (reviewed in Chargaff and Davidson, 1955 and in Pearse, 1960). 254-260 nanometer wavelength light is absorbed by nucleic acids whether they are bound to protein or not. This was known at the time and Caspersson recognized it, to his credit. The first reliable staining method for RNA was developed by Brachet, employing the dye pyronin G (reviewed in Brachet, 1957). The subsequent use of radioisotopic labeling of RNA in cells followed by autoradiography constituted a major advance for identifying intracellular sites of RNA synthesis, for example the nucleolus (Vincent and Miller, 1966). The ingenious application of nucleic acid hybridization to fixed cells by Joseph Gall and colleagues added an exciting new tool as the detection of RNA *in situ* entered its modern era (Gall and Pardue, 1969).

In the 1970s and 1980s, the microinjection of fluorescently labeled proteins into cells became a highly successful method in cell biology (Wang, 1989). In 1990, a graduate student in the author's laboratory, Jin Wang (no relation to the author cited above), asked whether fluorescent RNA might be injected into cells in the same way, to identify sites of localization or dynamics of translocation within the living

cell. He succeeded in this endeavor beyond even his own optimistic hopes (Wang, Cao, Wang and Pederson, 1991), and this approach, termed "Fluorescent RNA Cytochemistry, has become a valuable tool in the study of RNA localization and dynamics in living cells. This article describes this approach in general terms, referring to previously published methods articles for technical details. The notions of using Fluorescent RNA as either a *stain* or a *tracer* are discussed, and some examples of the application of Fluorescent RNA Cytochemistry in mammalian cells and frog oocytes are presented. A previous publication (Pederson, 2001) summarized all of the current methods for following RNA in living cells, while this article addresses Fluorescent RNA Cytochemistry in particular.

Materials and Methods

Two articles have been published that describe, in bench-level detail, the preparation of fluorescent RNA, its microinjection into cells and the microscopy systems and image acquisition software employed in the author's laboratory (Jacobson and Pederson, 1997; Jacobson, Pederson and Wang, 1998). Additional details pertinent to the present article are presented in the figure legends. In some experiments altered RNAs are generated by transcription from mutated DNA templates constructed by standard mutagenesis and recombinant DNA methods. Immunostaining of cells is performed as previously detailed (Jacobson, Cao, Wang and Pederson, 1995.) The NRK line of rat fibroblasts used in most of these studies in the author's laboratory and their growth and preparation for microinjection have been described (Wang, Cao, Wang and Pederson, 1991).

Results and Discussion

Making fluorescent RNA

There are two methods for producing fluorescent RNA for microinjection into cells. In one, a cloned DNA template for the particular RNA is transcribed by a promoter-suitable RNA polymerase in the presence of a fluorescently-labeled ribonucleoside triphosphate, together with all four unlabeled ribonucleoside triphosphates (e.g. Lange, Borovjagin and Gerbi, 1998). Alternatively, the desired RNA can be transcribed so as to contain a modified uridine nucleotide, 5-[3-aminoallyl]-UTP, at about one in every ten uridine positions. This allows post-transcriptional coupling of a fluorescent reporter to this incorporated nucleotide (Jacobson and Pederson, 1997; Jacobson, Pederson and Wang, 1998). In both methods, the level of substitution of fluorescent groups into the RNA can be controlled. In the author's laboratory, we usually introduce one fluorescent group every ~40 nucleotides in the RNA chain, having found that this typically results in readily detectable intracellular fluorescence and yet is a level of substitution that does not alter the properties of the fluorescent RNA relative to its endogenous RNA counterparts (Jacobson and Pederson, 1997).

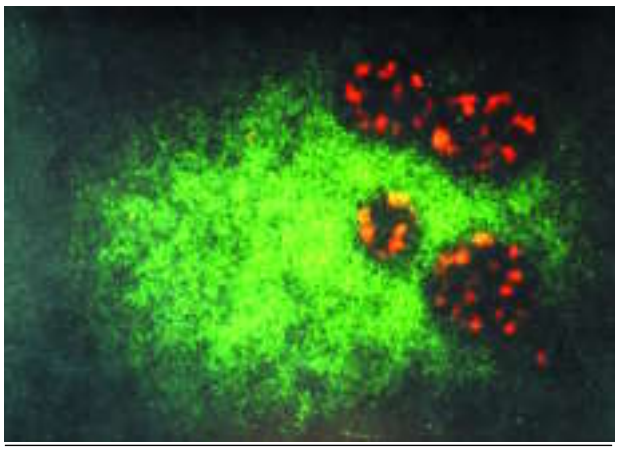

Figure 1. Rapid nucleolar localization of fluorescent RNase P RNA after microinjection into the nucleus. Human RNase P RNA was transcribed in the presence of 5-(3-aminoallyl)-UTP and recovered by ethanol precipitation. It was then coupled with tetramethylrhodamine-5-isothiocyanate and microinjected into the nucleus of NRK fibroblasts together with fluorescein-labeled dextran. This cell was imaged in the living state 4 minutes after microinjection.

RNA traffic into the nucleolus

Figure 1 shows an example of the application of Fluorescent RNA Cytochemistry in the case of a small RNA molecule called RNase P RNA, which is part of a ribonucleoprotein enzyme involved in transfer RNA 5′ end processing (Altman, 1990). Four minutes after microinjecting rhodamine-labeled RNase P RNA into the nucleus of a NRK fibroblast it became exclusively localized in the nucleoli, as shown by the red signal in the figure. The surrounding nucleoplasm was decorated green in this experiment by the co-injection into the nucleus of fluorescein-labeled dextran. In these studies the microinjections into the nucleus were aimed at nucleoplasmic sites not immediately adjacent to the nucleoli. The rapidity of this RNA's localization in the nucleoli

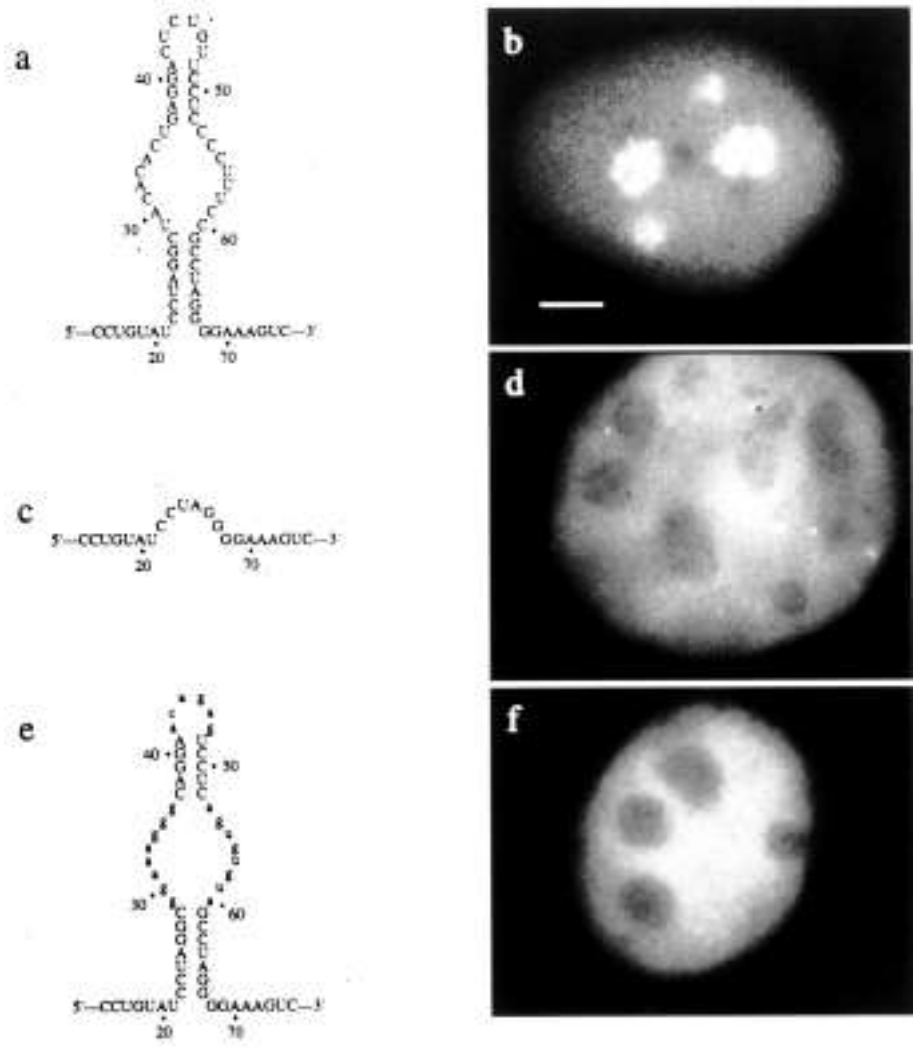

Figure 2. Sequence-dependent nucleolar localization of RNase MRP RNA. Wild-type or mutant versions of human RNase MRP RNA were transcribed, rhodamine-labeled and microinjected into the nucleus of NRK fibroblasts. The sequence elements of each RNA relevant to this experiment are shown in a, c and e. The intranuclear localization patterns of each of the three RNAs, obtained 2-5 minutes after microinjection, are shown in b, d and f. The bar in a is 5 mm. Reproduced from J Cell Biol 1995; 131:1649-58, by copyright permission of the Rockefeller University Press.

after microinjection into the nucleus is striking.

Figure 2 shows the results of an experiment with another species of RNA, called RNase MRP RNA. This RNA is also a component of a ribonucleoprotein enzyme, one involved in ribosomal RNA processing (Clayton, 1994). As can be seen in of Figure 2b, when a fluorescent version of this RNA was microinjected into the nucleus of a NRK fibroblast, it became localized in the nucleoli within one minute. This experiment serves to illustrate another important aspect of Fluorescent RNA Cytochemistry. Because the RNAs are generated by in vitro transcription from cloned DNA templates, one has the facile opportunity to generate altered RNAs for comparison. RNase MRP RNA contains a stem-loop element (Figure 2a) that is the binding site for a specific protein, called the To antigen (Yuan, Tan and Reddy, 1991). When this stem-loop was deleted (Figure 2c), the RNA did not display nucleolar localization (Figure 2d). When the stem-loop was mutated to have the same secondary structure but composed of a different nucleotide sequence (Figure 2e), this RNA also did not display nucleolar localization (Figure 2f), indicating that the actual nucleotide sequence of this region of the RNA is the critical factor for nucleolar targeting, not simply the stem-loop secondary structure. This study (Jacobson, Cao, Wang and Pederson, 1995) was the first to identify a nucleolar targeting element of an RNA.

Fluorescent RNA Cytochemistry can also be combined with the *in situ* detection of proteins by immunocytochemistry. As shown in Figure 3, rhodamine-labeled RNase MRP RNA in the nucleoli (left panel) and the protein fibrillarin detected by immunostaining (center panel) displayed spatially coincident localizations (right panel, in which the two sets of images have been computationally merged). This result identifies the site of RNase MRP RNA localization within the nucleolus as the dense fibrillar component (DFC), for which the protein fibrillarin is

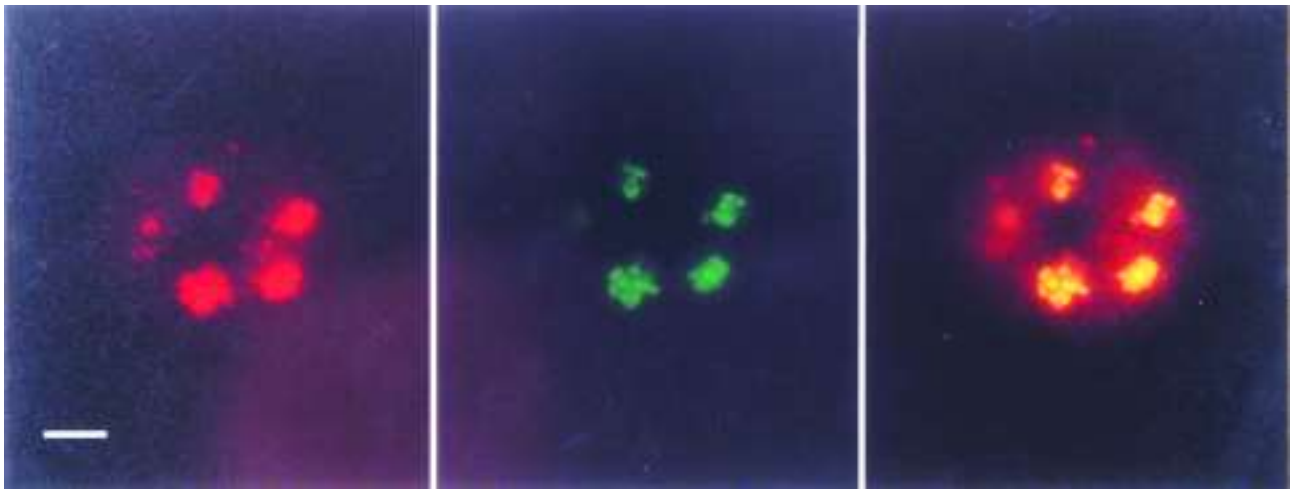

Figure 3. Combined fluorescent RNA localization and immunostaining. rhodamine-labeled human RNase MRP RNA was injected into the nucleus of NRK cells as in Figure 2b and the living cell was imaged one minute later (left panel), then fixed and immunostained with an antibody to fibrillarin (middle panel.) The right panel illustrates the computationally merged digital images. The bar in the left panel is 5 mm. Reproduced from J Cell Biol 1995; 131:1649-58, by copyright permission of the Rockefeller University Press.

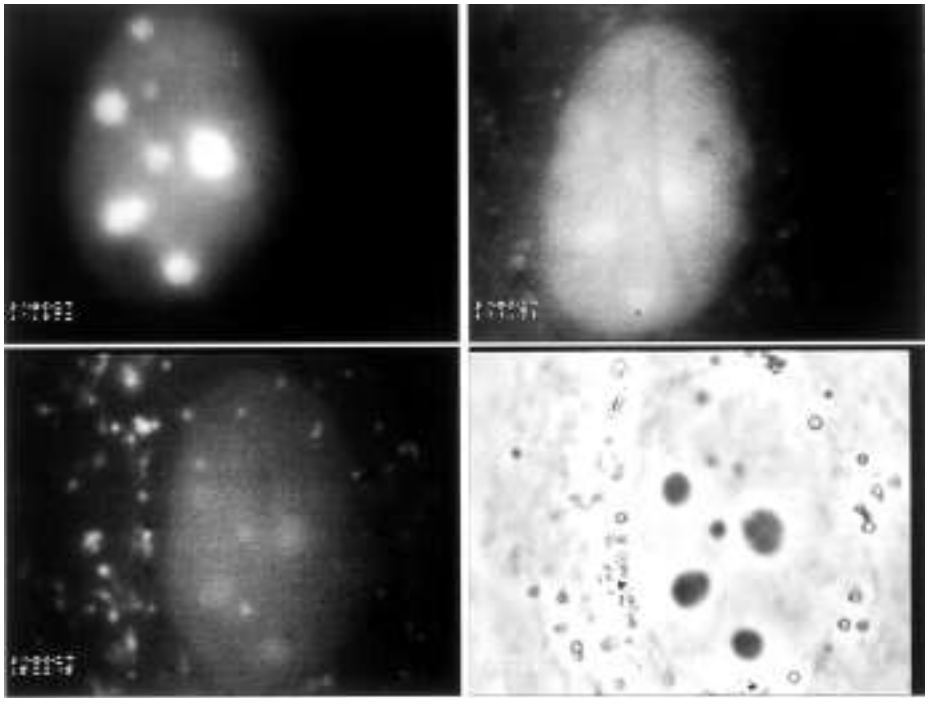

Figure 4. Transient localization of fluorescent signal recognition particle RNA in the nucleolus. Rhodamine-labeled canine signal recognition particle RNA was microinjected into the nucleus of NRK cells and imaged at 3 min. (upper left), 26 min. (upper right) or 66 min. (lower left) after microinjection. A phase contrast image of this cell is shown in the lower right. This figure is a modified version of one published in Proc Natl Acad Sci USA 1998; 95:7981-6 (Copyright the National Academy of Sciences, USA.

a fiduciary marker. (It is the specific localization of the other RNA, RNase P RNA (Figure 1) also in the dense fibrillar component that gives it the lobular sub-nucleolar appearance).

The "tracer" mode of fluorescent RNA cytochemistry

In some applications of Fluorescent RNA Cytochemistry, a microinjected RNA may traffic transiently though an initial intracellular site and then move and accumulate elsewhere with time. Such cases exemplify the tracer aspect of this method. An example of this behavior is shown in Figure 4. Here, a fluorescent version of the signal recognition particle (SRP) RNA has been microinjected into the nucleus of a NRK cell (Jacobson and Pederson, 1998). This RNA initially localizes in nucleoli (Figure 4, upper left). But unlike RNase P RNA (Figure 1) or RNase MRP RNA (Figure 2b and 3), which both localize stably in nucleoli, SRP RNA remains in nucleoli only transiently and then departs (upper right), followed by its appearance in the cyto-

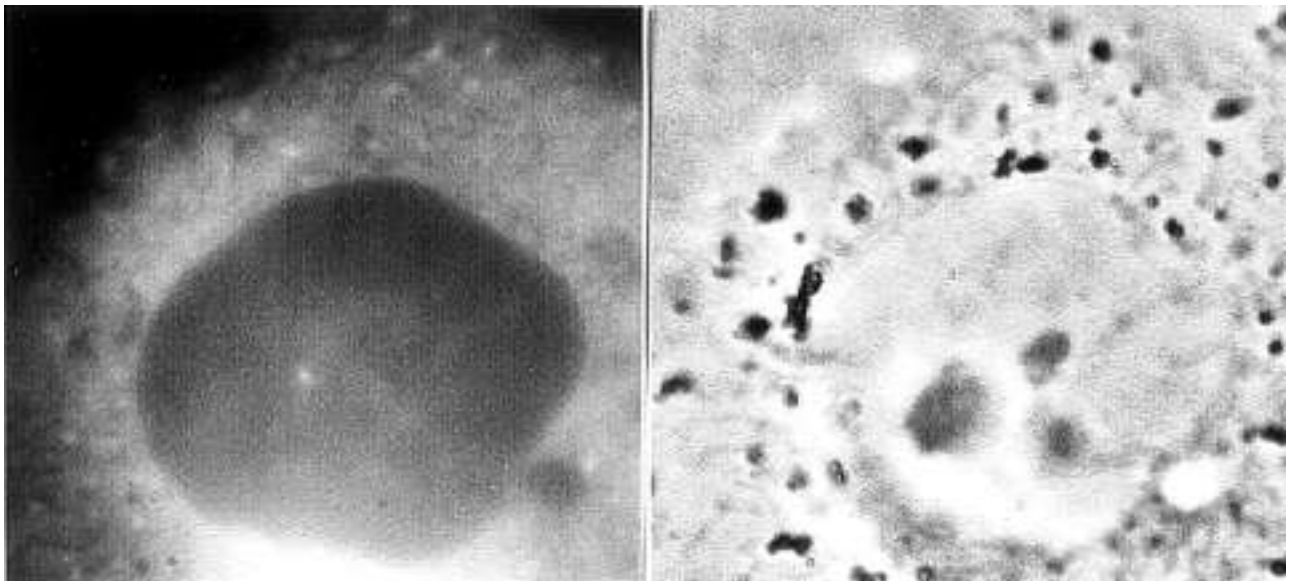

Figure 5. Cajal body localization of fluorescent U2 RNA following nuclear import. A rhodamine-labeled precursor of human U2 RNA was transcribed with a 7-methylguanosine cap at its 5' end and a 11 nucleotide extension at its 3' end (Kleinschmidt and Pederson, 1987). It was microinjected into the cytoplasm of NRK cells and followed as a function of time in the living cells. Left: fluorescence image 24 minutes after microinjection; the Cajal body localization was first evident at 9 minutes after microinjection (not shown.) Right: phase contrast image. The distinct focal site of U2 RNA within the nucleolus is a juxta-nucleolar structure identified as a Cajal body by immunostaining (see text.)

plasm (lower left). This temporal shift in localization is compatible with the presently understood intracellular pathway of signal recognition particle assembly and maturation, which appears to commence in the nucleolus prior to the export of nascent SRP to its sites of function in the cytoplasm (Walter and Johnson, 1994; Pederson and Politz, 2000). Another example of using Fluorescent RNA Cytochemistry to investigate the intracellular RNA translocation of RNA is in its application to studying the biosynthesis of the U1, U2, U4 and U5 spliceosomal small RNA species, which shuttle from nucleus to cytoplasm and back in their maturation (Zieve and Sauterer, 1990). These RNAs are exported from the nucleus as precursors elongated at their 3′ ends and then, in the cytoplasm, they assemble into a distinctive ribonucleoprotein form, and undergo 5′ end modification and 3′ end processing (Zieve and Sauterer, 1990; Kleinschmidt and Pederson, 1990; Huang and Pederson, 1999). In the case of human U2 spliceosomal RNA, the precursor that leaves the nucleus has an 11 nucleotide 3′ tail (Wieben, Nenninger and Pederson, 1985; Kleinschmidt and Pederson, 1987). Figure 5 shows an experiment in which a fluorescent version of this U2 RNA precursor molecule, carrying an 11 nucleotide 3′ tail, was microinjected into the cytoplasm of a NRK cell. It can be seen that after 24 minutes, some of the RNA has been imported into the nucleus and resides at a discrete focal site (left panel). This site occupies an intranuclear location close to a nucleolus (right panel, phase contrast image) and immunostaining experiments further identified these sites of newly imported U2 RNA as Cajal bodies, using an antibody for the Cajal body-specific protein, coilin (*not shown*) Cajal bodies are distinctive intranuclear domains that are thought to be staging areas for various gene expression molecules (Gall, 2000). The results shown in Figure 5 indicate that after completing its ribonucleoprotein assembly and 5′ and 3′ end processing in the cytoplasm, U2 RNA first enters Cajal bodies upon its return to the nucleus, before moving on to its ultimate destinations, the widespread nucleoplasmic mRNA splicing sites (Carmo-Fonseca et al., 1991.) The functional significance of this initial visit of newly imported U2 RNA to Cajal bodies is not known, but it represents a case in which Fluorescent RNA Cytochemistry reveals a provocative phenomenon with considerable clarity.

Moving RNA

From the very beginning of our work using fluorescent RNA in the nucleus, we were struck by the unanticipated high mobility of these RNAs. This stirred interest in the transport properties of RNA in the nucleus and led to studies tracking fluorescent

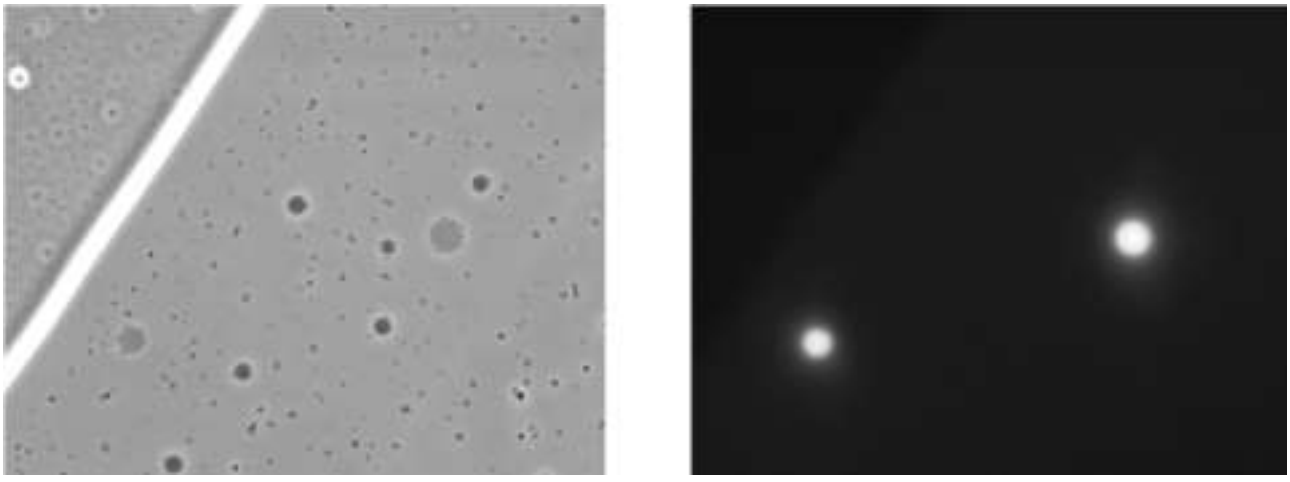

Figure 6. Localization of fluorescent U7 RNA in Cajal bodies of the germinal vesicle (nucleus) of *Xenopus* oocytes. Fluorescein-labeled *Xenopus* U7 RNA was microinjected into the cytoplasm of oocytes. After incubation overnight, the germinal vesicle was manually isolated under oil and gently compressed to spread the nuclear contents. Left: phase contrast image showing a region of nucleoplasm near the oil interface, with Cajal bodies and B-snurposomes evident. Right: fluorescence image, showing two Cajal bodies that have selectively taken up the U7 RNA. Reproduced from J Cell Biol 2003; 160:495-504, by copyright permission of the Rockefeller University Press.

RNA in the nucleus of mammalian cells using an important variation of Fluorescent RNA Cytochemistry, involving hybridization of photoactivatable oligonucleotide probes to endogenous RNAs (Politz, Tuft and Pederson, 2004). However, the intranuclear mobility of RNA can also be studied using microinjected fluorescent RNA in some cases. An example is a recent study by Handwerger, Murphy and Gall (2003). In these experiments, a fluorescently labeled version of the U7 small nuclear RNA was microinjected into the cytoplasm of oocytes of the amphibian *Xenopus laevis* (U7 RNA functions in the 3′ end processing of histone pre-mRNA in the nucleus). As can be seen in Figure 6, right panel, the fluorescent U7 RNA became selectively localized within Cajal bodies. Once this highly specific intranuclear localization of U7 RNA in the living oocyte had been established, it was possible to investigate the mobility of this RNA by the method of fluorescence recovery after photobleaching, or FRAP. This revealed a rapid U7 RNA nucleoplasmic movement, with an estimated diffusion coefficient of ~0.25 m^2/sec (Handwerger, Murphy and Gall, 2003). In contrast, the mobility of this RNA within the Cajal bodies themselves was observed to be considerably lower, displaying a maximum recovery time of ~35 min. This study illustrates how Fluorescent RNA Cytochemistry can exceed its original *stain* and intracellular *tracer* modes, to provide a way to study RNA molecular dynamics in living cells, complementing other approaches (Politz, Browne, Wolf and Pederson, 1998: Politz, Tuft and Pederson, 2004).

Summary

The foregoing examples illustrate how the introduction of fluorescent RNA molecules into cells can serve as an informative tool in gene expression research. Like most methods, Fluorescent RNA Cytochemistry has both advantages and disadvantages. The key advantage is that the information generated by this method is obtained in living cells. A potential disadvantage is that because the RNA molecules are doped by the attachment of fluorescent groups, they might behave differently in the cell relative to their natural counterparts, and careful controls and vigilance are continually warranted on this point. Fortunately, the hope that fluorescent RNAs introduced into cells are behaving correctly has been empirically demonstrated in many cases, often by comparing the behavior with that of the introduced RNA's endogenous counterparts as determined by RNA *in situ* hybridization. The other potential disadvantage of Fluorescent RNA Cytochemistry for mammalian cells, or other cells of similar size (but not amphibian oocytes) is that it requires intracellular microinjection, indeed intranuclear microinjection in many cases. Our experience is that the technique of intranuclear microinjection of cultured mammalian cells can be readily learned with practice and it has not seemed to have been an impediment to those investigators wishing to use this method. The effort required is actually no more time consuming than investigating and practicing the optimal fixation and staining of cells and tissue sections the author undertook back in his student days, when first trying cytochemistry

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