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SURVEY AND SUMMARY

Fluorescent RNA cytochemistry: tracking gene transcripts in living cells

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ABSTRACT

The advent of jellyfish green fluorescent protein and its spectral variants, together with promising new fluorescent proteins from other classes of the Cnidarian phylum (coral and anemones), has greatly enhanced and promises to further boost the detection and localization of proteins in cell biology. It has been less widely appreciated that highly sensitive methods have also recently been developed for detecting the movement and localization in living cells of the very molecules that precede proteins in the gene expression pathway, i.e. RNAs. These approaches include the microinjection of fluorescent RNAs into living cells, the *in vivo* hybridization of fluorescent oligonucleotides to endogenous RNAs and the expression in cells of fluorescent RNA-binding proteins. This new field of 'fluorescent RNA cytochemistry' is summarized in this article, with emphasis on the biological insights it has already provided. These new techniques are likely to soon collaborate with other emerging approaches to advance the investigation of RNA birth, RNA–protein assembly and ribonucleoprotein particle transport in systems such as oocytes, embryos, neurons and other somatic cells, and may even permit the observation of viral replication and transcription pathways as they proceed in living cells, ushering in a new era of nucleic acids research *in vivo*.

INTRODUCTION

Cytochemistry and histochemistry, the identification of the chemical nature and location of substances within cells and tissues, began to emerge about 150 years ago and came of age in the first half of the 20th century. The specificity of many of these staining reactions was validated through biochemical studies with defined substrate materials, as well as by enzymatic or chemical pre-depletion of the suspected target molecules in the cells or tissue sections being stained. For example, confidence in the Feulgen reaction for DNA, one of the most specific reactions in the history of cytochemistry, was greatly bolstered when Moses Kunitz (Rockefeller Institute) crystallized pancreatic DNase and this preparation was shown to abolish staining. Shortly thereafter, Hans Ris with Alfred Mirsky (Rockefeller) and Hewson Swift with Arthur Pollister (Columbia) used Feulgen staining and microspectrophotometry to show that the DNA content of the gametes of several vertebrate species was half that of the animal's somatic cells, a powerful epistemological step in the acceptance of the idea that the gene was DNA.

Histochemistry and cytochemistry were at that time, and still are, impressive arts when skillfully practiced by experts. Nonetheless, these methods employ slices of chemically fixed (or sometimes frozen) biological material. Chemical fixation is understandable given the roots of histochemistry in the field of pathology (being mindful of what an unfixed piece of tissue starts to smell like a few hours after excision). And yet, there has always been another way.

In a then-famous experiment 161 years ago, the physiologist Claude Bernard injected a dog intravenously with iron lactate and potassium ferrocyanide, resulting in the formation of a colored reaction product (Prussian blue) at sites of very low pH in the body, i.e., the gastric mucosa (1). Although color reactions with plant extracts had been described almost 2000 years earlier (2,3), Bernard's experiment was probably the first report of a histochemical reaction in living tissue. Ninety years later a biochemist in Woods Hole (USA) decided to extract rabbit psoas muscle with glycerin. To this preparation Albert Szent-Györgyi added ATP and thus gave birth to a profound revolution. Somewhat later, H.G.Callan in Scotland and Jan-Erik Edström in Sweden prepared beautiful chromosomes from living cells for the fixative-prone cytologists of the day to admire with great envy. Thus it came to be that two theaters of cell research emerged more or less contemporaneously but with minimal communication and cross-pollination: on the one hand histochemistry, employing fixed cells or tissue, and on the other a school of cell physiology employing living or gently extracted material.

RNA CYTOCHEMISTRY *IN VIVO*

As a first year graduate student in 1963 I encountered the book 'Biochemical Cytology' by the Belgian cytologist and embryologist Jean Brachet (4), which conveyed that Torbjörn Caspersson in Stockholm had used ultraviolet cytophotometry

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and RNase digestion to show that the classical cytoplasmic basophilia seen by histochemistry was due to RNA, and that shortly thereafter Brachet had also identified the nucleic acid of the cytoplasm as RNA. Both of these pioneers had bravely advanced the notion that the level of cytoplasmic RNA was related to cell growth and differentiation and thus to the expression of genes. In retrospect, it seems quite remarkable that such a profound idea, coming almost a decade before the discovery of ribosomes and messenger RNA, had arisen in the 'simple' field of cytochemistry, i.e. non-genetic/non-molecular biology. The modern science of RNA cytochemistry began when Brachet, Caspersson and Edström perfected the cytochemical and microanalytical investigation of intracellular RNA. Now, in the post-modern era, fluorescent RNA reigns.

The initial work in this new field involved the microinjection of fluorescent RNA into mammalian cells (5). Several intron-containing pre-mRNAs were observed to become localized at discrete intranuclear foci within 20–30 min after microinjection into the nucleus, whereas intron-lacking mRNA or other, non-mRNAs, did not show such localization (5). By carrying out immunostaining with antibodies specific for mRNA splicing factors, it was found that the intranuclear sites of fluorescent pre-mRNA localization coincided with previously described depots at which these mRNA splicing factors are concentrated, known cytologically as intrachromatin granule clusters and, more popular recently, as 'speckles' (5,6).

Fluorescent RNA microinjection is both literally 'cytochemistry' and more. On the one hand the introduced fluorescent RNA can bind to high affinity sites in living cells, just like dyes such as pyronin, cresyl violet or azure B bind to RNA itself in the basophilic cytoplasm of fixed tissue in the days of classical histochemistry. But fluorescent RNA can also be a 'tracer' in which it can reveal, as a function of time after administration, a pathway of intracellular transit in the living cell.

The nucleus microinjection of fluorescent RNAs was subsequently employed to define the behavior of several small RNAs that localize (permanently or transiently) in the nucleolus (7–12). Among other things, these studies revealed the first nucleolus-targetting element for an RNA (7). In addition, one of the RNAs that this work revealed to have a nucleolar association is the signal recognition particle RNA (12), a finding that catalyzed new ideas about the nucleolus as a plurifunctional organelle (13–16) and that has led to further studies on SRP components in the nucleolus (17,18). Meanwhile, numerous other studies have employed microinjection of fluorescent RNA into the nucleus to investigate the dynamics and localization of additional nucleolar RNAs (19–26), as well as to further investigate the intranuclear localization of splicing-competent versus splicing-deficient mRNAs (27). Fluorescent RNA microinjection has also advanced our understanding of mRNA traffic in the cytoplasm, particularly in neuronal cells (28–30).

WATCHING POLY(A) RNA MOVE IN THE NUCLEUS

In all the studies involving microinjection of fluorescent RNA into the nucleus, the RNA was very soon thereafter observed at its sites of intracellular concentration, e.g. the nucleolus for RNase MRP RNA, RNase P RNA, SRP RNA and various other small nucleolar RNAs (7,8,11,12,19–25) or at interchromatin granule clusters for pre-mRNA (4,27). However, it was not feasible in any of these numerous studies to capture the actual movement of the RNAs, in part because it was so fast.

In an important advance, Politz *et al.* (31) showed that oligo(dT) introduced into rat myoblasts becomes hybridized to poly(A) RNA, based on an *in situ* oligo(dT)-primed reverse transcription assay (31,32). This was the first compelling demonstration that an oligonucleotide introduced into living cells actually hybridizes to complementary RNA sequences. Subsequently, fluorescence correlation spectroscopy (FCS) revealed that the fluorescent oligo(dT) inside the nuclei of living rat myoblasts moved at rates expected if the probe were hybridized to poly(A) RNA molecules that were moving by diffusion (33). Moreover, the movement was unaffected when ATP levels were pharmacologically reduced (33). Fluorescence recovery after photobleaching (FRAP), an established method for measuring the diffusion of molecules in living cells, gave results very similar to those determined by FCS microscopy (33). These findings bolstered confidence in the RNA-hybridized state of the fluorescent oligo(dT). However, although these FCS and FRAP studies provided a rigorous interrogation at the microscopic physical scale of molecular dynamics, the goal of actually watching RNA movement in living cells, in real time, remained a challenge.

The next step in the fluorescent RNA cytochemistry field involved 'caged' fluoresceins, a family of compounds initially synthesized by Timothy Mitchison (34). In these caged fluoresceins, two *O*-nitrobenzene groups attached via ether linkages lock the fluorescein into its non-fluorescent tautomer (35). These caging groups can be split off by 360 nm photolytic cleavage, resulting in native fluorescein (λ_{ex} = 418 nm).

Politz *et al.* (36) found that oligo(dT) labeled with this caged fluorescein had cellular uptake and poly(A) RNA hybridization properties similar to (non-caged) fluorescent oligo(dT). The intranuclear movement of poly(A) RNA was then investigated, using caged fluorescent oligo(dT) followed by localized uncaging in a defined intranuclear volume by 360 nm light brought in through a 1.5 μm aperture in the microscope condenser from an argon multiline laser, so that the uncaged probe bound to poly(A) RNA could be tracked as a bright signal moving out into (initially) dark surrounding regions of the nucleus (36). These experiments were made possible by a very rapid image acquisition fluorescence microscopy technology (37,38). It was found that the poly(A) RNA signal moved out from the uncaging site in all directions and eventually occupied all of the nucleus except for the nucleoli, from which it was excluded. When the DNA in these cells was stained with Hoechst 33342 and imaged relative to the distributed poly(A) RNA, it was found that the majority of the RNA was located between the interphase chromosomes. These results demonstrated that poly(A) RNA moves randomly in the nucleus. Additional data indicated that this movement is independent of temperature between 23 and 37°C, which is compatible with a diffusion-based process in which metabolic energy is not limiting (36), and supports the FCS and FRAP results (33).

The nuclear poly(A) RNA tracked in these experiments contains multiple biosynthetic classes, including pre-mRNA as well as some nucleus-restricted poly(A) RNAs of unknown function. Therefore it was not certain whether the observed random, diffusion-like movement reflected the productive pre-mRNA→mRNA pathway, the behavior of nucleus-restricted poly(A) RNAs, or both. However, a subsequent study of the

intranuclear movement of a specific, well-characterized pre-mRNA, which undergoes splicing and is exported to the cytoplasm as a *bona fide* messenger RNA, revealed that this transcript moves away from the gene in a random walk fashion characteristic of diffusion (39,40). Additional evidence for export of a specific mRNA over the entire array of nuclear pores has been reported (41,42). The picture that emerges is one of processed mRNAs moving out into the interchromatin space by diffusion, then encountering specific binding proteins that create an 'export me' ribonucleoprotein signature (42), with the resulting RNP complexes then stochastically encountering the (highly complex) nucleoplasmic side of the nuclear pore machinery (43). It is to be noted that several recent studies on the movement of proteins within the nucleus have also revealed a pattern of rapid, random, ATP-independent motion indicative of diffusion (44,45), suggesting that whatever organized structure exists in the interchromatin space (44,46,47) it does not appear to contravene the movement of RNA or proteins as a process that appears to be mechanistically one of diffusion, however constrained by the plausibly crowded intranuclear environment.

A third approach recently introduced to track the movement and localization of RNA in living cells involves the expression of a green fluorescent protein (GFP)-tagged protein that binds to a specific RNA target sequence (48). The chief appeal of this method is that it lends itself to the now quite routine practice of expressing GFP fusion proteins in a wide variety of cells. This method was initially applied to track a specific mRNA in living yeast cells (48) and has more recently been applied to follow mRNA transport in cultured rat hippocampal neurons (49). Each of these approaches has inherent advantages and limitations. Microinjection of fluorescent RNA requires a certain degree of manual adroitness, although this skill is readily acquired by most who undertake it. The use of caged fluorescent oligonucleotides to track RNA depends on good cellular uptake and productive hybridization, and requires ultra-fast image acquisition microscopy expertise (37,38). The method, based on GFP protein binding to RNA targets, requires that the GFP protein expression level operates in a RNA titration mode (i.e. the method is compromised if there is an excess of the GFP protein not bound to RNA), although this has been addressed in a subsequent version of the method that has been developed (50). Moreover, in each of these methods the fact that the reporting RNA is derivatized must be borne in mind. With microinjected RNAs there is a fluorochrome every 40 nt or so. With caged fluorescent oligonucleotides the targetted RNA has short double-stranded regions of hybridized oligo beyond the RNA's inherent secondary structure and it is possible that this exerts some influence on the observed movement. In the GFP protein-based method, the RNA target contains six or more inserts of a bacteriophage RNA sequence and thus potentially has multiple RNA binding proteins attached, thus itself constituting a ribonucleoprotein particle, although for some purposes the number of protein binding sites can be reduced (50,51). Thus, these three methods involve increasing chemical derivatization and attendant molecular mass in the order they are mentioned above, and yet have decreasing experimental complexity in the same stated sequence.

PROSPECTS

The three new approaches summarized in this review, collectively termed 'fluorescent RNA cytochemistry', have applications both in the nucleus and the cytoplasm. In cells like oocytes and neurons that have to move some RNAs a long distance from the nuclear transcription site, *in situ* hybridization studies have been important but can only tell us so much, and here fluorescent RNA cytochemistry offers great promise. These new methods are also attractive to contemplate as regards viral RNAs, especially those transcribed by paramyxoviruses, for example, influenza, diplornaviruses (e.g. reovirus) and lentiviruses (e.g. HTLV-I and HIV), as well as many plant viruses, in all of which cases our knowledge of the intracellular localization and transport of the viral genomic RNA and mRNAs remains incomplete, often due to the limitations of cell fractionation experiments. The application of these fluorescent RNA methods to oocytes, embryos and other specialized cells such as neurons, virus-infected cultured cells or even biopsied patient cells has the potential not only to corroborate static RNA localization results based on *in situ* hybridization but also to open the door to kinetic and molecular dynamic features in the context of the living state. Moreover, it may soon be possible to probe *in vivo* the molecular association of fluorescent RNAs with their protein binding partners, the latter painted with one of the many colors on the fluorescent protein polychromatic palette now available (52–56). One promising approach is a method with origins prior to the birth of allostery (57) and subsequently refined for cell biology (58–60), namely fluorescence resonance energy transfer, or FRET. The opportunity to apply RNA–protein FRET to actually watch a gene expression or viral replication pathway unfold in living cells may soon be at hand. We are thus witnessing a new era in the study of RNA in living cells. All this has come down to us from the venerable fields of cytology, cytochemistry and histochemistry, and is fueled now by an exciting sense of contemporary congression between biophysics and cell biology (44), in which it will become possible for gene expression in living cells to be studied as molecular dynamics.

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