Methods xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

# The origin of in situ hybridization – A personal history

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ARTICLE INFO

*Article history:*
Received 12 October 2015
Received in revised form 27 November 2015
Accepted 29 November 2015
Available online xxxx

*Keywords:*
Autoradiography
Gene amplification
In situ hybridization
Nucleolus
Oocyte
Ribosomal RNA

ABSTRACT

In situ hybridization is the technique by which specific RNA or DNA molecules are detected in cytological preparations. Basically it involves formation of a hybrid molecule between an endogenous single-stranded RNA or DNA in the cell and a complementary single-stranded RNA or DNA probe. In its original form the probe was labeled with $^{3}H$ and the hybrid was detected by autoradiography. The first successful experiments in 1968 involved detection of the highly amplified ribosomal DNA in oocytes of the frog *Xenopus*, followed soon after by the reiterated "satellite DNA" in mouse and *Drosophila* chromosomes. Fluorescent probes were developed about ten years later.



My lifelong interest in giant chromosomes and what they might tell us about gene structure and function began about 1950, when I started graduate work at Yale in the laboratory of Donald Poulson. Poulson was a *Drosophila* geneticist who had trained at CalTech when T.H. Morgan, A.H. Sturtevant, and C.B. Bridges were still establishing *Drosophila* as the key organism for genetic analysis. Bridges had just completed his maps of the giant polytene chromosomes found in the larval salivary glands. These chromosomes were called polytene (many stranded) because they consisted of hundreds to thousands of copies of individual chromosomes lined up parallel to one another. With Poulson's help I made "squash" preparations of *Drosophila* polytene chromosomes and familiarized myself with the exquisite banding pattern that allowed one to map the positions of individual genes. In the end, however, I could not think of anything new to do with these chromosomes.

As luck would have it, I stumbled onto the fact that giant chromosomes of another sort are found in the oocytes of frogs and salamanders. These are the so-called lampbrush chromosomes (LBCs), discovered in the late 19th century, but largely overlooked by cell biologists in the decades following their first description [1]. They were given the name lampbrush because of their fuzzy appearance, which was reminiscent of the brushes used at that time to clean the chimneys of oil lamps. I studied these chromosomes for my thesis, making use of the important discovery by William Duryee [2] that the amphibian oocyte nucleus and its giant chromosomes could both be isolated by hand from living oocytes. I was lucky that commercial phase contrast microscopes had just been introduced and so I was able to examine "living" LBCs for the first time at high magnification. About this time an investigator in Scotland, H.G. "Mick" Callan, also realized the advantages of LBCs. We quickly became friends and eventually collaborators. As we slowly learned, LBCs are the very opposite of polytene chromosomes, despite their enormous size. They are in prophase of the first meiotic division and each consists of exactly four chromatids, as in any other meiotic chromosome. What gives them their unique appearance are hundreds of looped out regions, where sister chromatids are not closely associated with each other. It gradually became clear from experiments that Mick Callan and I carried out that the paired loops are regions of active RNA synthesis.

After completing my Ph.D. at Yale, I took a position as Instructor at the University of Minnesota, where I remained for the next 11 years, rising up the academic ladder. Convinced that the bands of polytene chromosomes and the paired loops of LBCs corresponded to individual genes or small clusters of genes, I spent many hours wondering how we could use these chromosomes to identify specific gene loci. Sometime around this time I learned of an important paper recently published by two immunologists, Albert Coons and Melvin Kaplan [3]. They showed that individual proteins could be localized at the cellular level using an antibody coupled to fluorescein isocyanate. Of course, there were no commercial fluorescent antibodies available, so anyone wishing to make use of this new technique was faced with the rather daunting task of synthesizing their own probes. Moreover, epifluorescence microscopes were a thing of the future, so one also had to put together one's own microscope using a wide-angle condenser designed for dark-field observations. Such a condenser was oiled

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http://dx.doi.org/10.1016/j.ymeth.2015.11.026
1046-2023/

to the bottom of the microscope slide on which the preparation was mounted. A mercury arc fitted with a deep blue filter supplied the necessary light for excitation of the fluorophore and a yellow filter in the eyepiece allowed one to observe the yellow-green fluorescence.

I decided to test the hypothesis that individual loops of the LBCs might carry gene-specific proteins that could be detected by the marvelous new technique of Coons and Kaplan. So I put together a fluorescence microscope and convinced a friend in the Chemistry Department to synthesize some fluorescein isocyanate for me (it was a dangerous synthesis involving phosgene, among other things). Antibody production was an equally daunting task for someone with no training in immunology. Fortunately, a graduate student in our department was making antibodies in chickens, so I was able to make use of his expertise (and chickens). I had no good idea what proteins to test, so I isolated and solubilized a large number of giant nuclei from salamander oocytes, injected them into chickens, and collected the serum. Equally naively, I conjugated the whole serum with fluorescein and applied the mixture to LBC preparations. As I remember – I cannot find any notes from these experiments – I never saw anything of interest. The fact that no publications resulted from this rather heroic foray into immunofluorescence is further evidence that there were no significant results. Nevertheless, these studies familiarized me with some basics of immunology and gave me a good working knowledge of fluorescence microscopy, both of which would be valuable in the future.

In 1963 I returned to Yale for a sabbatical year. As things worked out, I was offered a professorship there, and I remained at Yale for the next 20 years. It soon became clear that I could not continue to make meaningful contributions to cell biology without learning the new techniques for analyzing RNA and DNA – what came to be called molecular biology. Over the next few years I continued to study the amphibian oocyte, but now with an emphasis on the molecular aspects of transcription. In those days – a decade before molecular cloning – the easiest molecules to study were the two RNA subunits of the ribosome, 18S and 28S ribosomal RNA (rRNA). Despite technical limitations, several groups made rapid progress in understanding these molecules in a variety of organisms. From the standpoint of cell biology, the most important finding was that newly synthesized rRNA appeared first in the nucleolus and that the genes coding for that RNA (rDNA) were reiterated and associated with the nucleolus in amphibians [4,5] and in *Drosophila* [6]. These studies tied the new molecular information to cytological observations made three decades earlier by Barbara McClintock [7]. Somewhat oversimplified, McClintock's "nucleolar organizer" corresponded to the rDNA in the chromosome, whereas the nucleolus itself represented the material being organized – rRNA and associated proteins.

This elegant understanding of the nucleolus and its important role in cell biology posed significant problems for those of us studying the amphibian oocyte. Since the 19th century it had been known that the gigantic nucleus inside the amphibian oocyte contained not one or two nucleoli but hundreds. Even more perplexing, these nucleoli were not attached to the chromosomes, but were localized primarily around the periphery of the nucleus near the nuclear envelope. How could they be involved in rRNA synthesis if they were not at the rDNA loci on the chromosomes? The easiest resolution of this dilemma would have been that these extrachromosomal "nucleoli" were not true nucleoli at all. That is, that they were not involved in rRNA synthesis, but represented some other kind of nuclear body, of which several were already known.

As things turned out, however, the multiple nucleoli *are* involved in rRNA synthesis. Each extrachromosomal nucleolus contains a small amount of rDNA, identical to the rDNA at the nucleolus organizer on the chromosomes. The history of "gene amplification," as this phenomenon was named by Oscar Miller in 1965 [8], is quite complicated. In fact, gene amplification had been clearly described and correctly interpreted many years before, but had been largely ignored or forgotten [9,10]. What was new was not the phenomenon, but rather the identification of rDNA as the specific molecule involved in amplification. Briefly, Oscar Miller [8] and Jim Kezer (cited in [11]) showed by DNase digestion that the free nucleoli in amphibian oocytes contained DNA, thereby confirming earlier claims based on staining with the Feulgen reagent. That this DNA was rDNA was demonstrated independently by me [12] and by Don Brown and Igor Dawid [13]. The most important fact for the subsequent development of in situ hybridization was the realization that the extra rDNA is synthesized early in meiosis and accumulates as a prominent cap on one side of the nucleus (Fig. 1). In other words, rDNA amplification is complete *before* the oocyte begins its growth phase and *before* the multiple nucleoli are formed.

Although I spent most of my time on the molecular biology of rRNA, I did not forget my hope to develop some way to visualize the genes on polytene and lampbrush chromosomes. Fluorescent antibodies had been a flop, but now the thought of nucleic acid hybridization seemed a possibility. The melting and reassociation of DNA was demonstrated in the early 1960s as well as the hybridization of RNA to complementary DNA sequences. Many of these experiments were done with $^{32}$P-labeled molecules, the reassociated molecules or hybrids being detected by scintillation counting. One particularly useful technique was filter hybridization. Here one attached denatured, single-stranded DNA to a nitrocellulose filter and then incubated the filter in a solution of radioactive RNA. This technique, developed especially by Gillespie and Spiegelman [14], proved extremely useful for molecular analysis of RNA-DNA hybrids. I used this technique in many of my experiments on rRNA and it occurred to me that it should be possible to adapt the method to cytological preparations. The idea was to make a flat tissue squash, denature the DNA in the nuclei on the slide, and then hybridize with $^{3}$H-labeled rRNA. Detection would be by autoradiography rather than scintillation counting. I had done a good deal of autoradiography, so that didn't pose any prob-

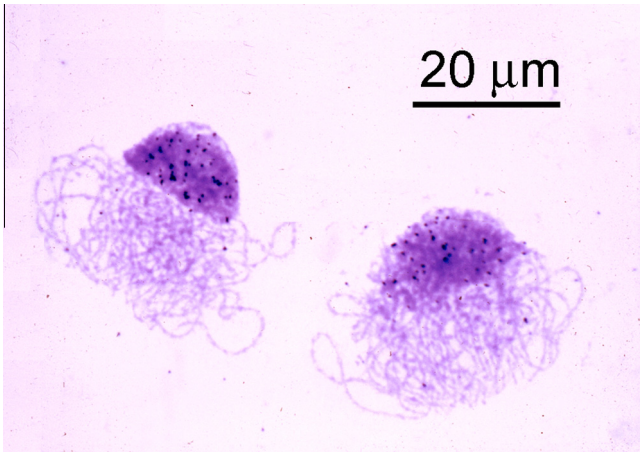


**Fig. 1.** Autoradiograph of two oocyte nuclei in the pachytene stage of meiosis from a *Xenopus* ovary. In addition to the chromosomes in the characteristic "bouquet" arrangement, each nucleus contains a massive cap of amplified rDNA. The black dots over the cap are silver grains in the autoradiographic emulsion that covers the preparation. However, this is *not* an in situ hybridization. The silver grains were produced by radioactivity from $^{3}$H-thymidine incorporated into the DNA of these nuclei. The grains indicate that the rDNA in the cap is still replicating at the pachytene stage, long after the chromosomes have ceased replication.

Please cite this article in press as: J.G. Gall, Methods (2015), http://dx.doi.org/10.1016/j.ymeth.2015.11.026