Fluorescence in situ hybridization: past, present and future

Jeffrey M. Levsky and Robert H. Singer*

Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA
*Author for correspondence (e-mail: rhsinger@aecom.yu.edu)

Journal of Cell Science 116, 2833-2838 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00633

Summary

Fluorescence in situ hybridization (FISH), the assay of choice for localization of specific nucleic acids sequences in native context, is a 20-year-old technology that has developed continuously. Over its maturation, various methodologies and modifications have been introduced to optimize the detection of DNA and RNA. The pervasiveness of this technique is largely because of its wide variety of applications and the relative ease of implementation and performance of in situ studies. Although the basic principles of FISH have remained unchanged, high-sensitivity detection, simultaneous assay of multiple species, and automated data collection and analysis have advanced the field significantly. The introduction of FISH surpassed previously available technology to become a foremost biological assay. Key methodological advances have allowed facile preparation of low-noise hybridization probes, and technological breakthroughs now permit multi-target visualization and quantitative analysis – both factors that have made FISH accessible to all and applicable to any investigation of nucleic acids. In the future, this technique is likely to have significant further impact on live-cell imaging and on medical diagnostics.

Key words: FISH, DNA, RNA, Fluorescence, Imaging, Microscopy, Hybridization, Computer image processing

Early years

The broader historical setting for the development of cytochemical techniques in general is extensively and excellently reviewed elsewhere (van der Ploeg, 2000). We present a much-abridged history to describe the introduction, development and maturation of fluorescence in situ hybridization (FISH) specifically. In brief, the earliest histochemistry techniques consisted of the use of different sorts of natural and synthetic dyes to stain cellular structures and sub-cellular accumulations. These compounds were generally non-specific because they had affinities for certain general categories of molecules, be they basic proteins, nucleic acids, lipids or carbohydrates. Even the more specific stains for cellular accumulations and macromolecular complexes such as hemosiderin, amyloid, elastin and reticular fibers were not generally applicable to investigation of all the biomolecules of interest. The ability to detect specific molecular identities was first demonstrated using antigen-antibody interactions. Early in the 1940s, antibodies were conjugated to fluorochromes without loss of their epitope-binding specificity. Decades later, the first antibody-dependent fluorescent detection of nucleic acid hybrids was achieved (Rudkin and Stollar, 1977); however, this technology was soon replaced by the advent of fluorescent nucleic acid probes. The earliest in situ hybridizations, performed in the late 1960s, were not fluorescent at all, but rather utilized probes labeled with radioisotopes. Techniques not employing fluorescence, such as enzyme-based chromogenic reporters (reviewed by Hougaard et al., 1997) and gold-based probe systems used in electron microscopy (reviewed by Puvion-Dutilleul and Puvion, 1996) are each fields in their own right. Owing to space limitations, we cannot expand upon these topics further here; we focus our discussion specifically on FISH.

FISH for visualization of nucleic acids developed as an alternative to older methods that used radiolabeled probes (Gall and Pardue, 1969). Early methods of isotopic detection employed non-specific labeling strategies, such as the random incorporation of radioactive modified bases into growing cells, followed by autoradiography. Several drawbacks of isotopic hybridization inspired the development of new techniques. First, the very nature of radioactive material requires that the probe is unstable; the isotope decays over time and so the specific activity of the probe is not constant. Second, although sensitivity of radiography is generally high, resolution is limited. Third, long exposure times are often required to produce measurable signals on radiography film, delaying results of the assay. Fourth, radiolabeled probe is a relatively costly and hazardous material and it must be transported, handled, stored and disposed of in accordance with regulations. FISH allowed significant advances in resolution, speed and safety, and later paved the way for the development of simultaneous detection of multiple targets, quantitative analyses and live-cell imaging.

The first application of fluorescent in situ detection came in 1980, when RNA that was directly labeled on the 3′ end with fluorophore was used as a probe for specific DNA sequences (Bauman et al., 1980). Enzymatic incorporation of fluorophore-modified bases throughout the length of the probe has been widely used for the preparation of fluorescent probes; one color is synthesized at a time (Wiegant et al., 1991). The use of amino-allyl modified bases (Langer et al., 1981), which could later be conjugated to any sort of hapten or fluorophore,

was critical for the development of in situ technologies because it allowed production of an array of low-noise probes by simple chemistry. Low probe specific activity prevented the assessment of nucleic acids with low copy number by FISH. Methods of indirect detection allowed signal output to be increased artificially by the use of secondary reporters that bind to the hybridization probes. In the early 1980s, assays featuring nick-translated, biotinylated probes, and secondary detection by fluorescent streptavidin conjugates were used for detection of DNA (Manuelidis et al., 1982) and mRNA (Singer and Ward, 1982) targets. Approximately a decade later, improved labeling of synthetic, single-stranded DNA probes allowed the chemical preparation of hybridization probes carrying enough fluorescent molecules to allow direct detection (Kislauskis et al., 1993). Many variations on these themes of indirect and direct labeling have since been introduced, giving a wide spectrum of detection schemes from which to choose; the specifications, sensitivity and resolution of these techniques are well described elsewhere (Raap, 1998).

Coming of age

Although the number of methods of detection has increased, the types of target have become quite varied. One might say that targets progressed from somewhat large to larger and then back towards small. Early probes produced from clones had to be large because they were sparsely labeled in order to allow specific hybridization. Probes also had to be large because of the methods used for their synthesis and purification. Hybridization probes were prepared by growth in a vector, nick-translation, in vitro transcription, or random-primed DNA synthesis. The large probes, however, often contained repeat sequences that made them prone to high background fluorescence. Suppression hybridization by pre-treatment with unlabeled nucleic acids to compete for non-specific sites of binding overcame this problem (Landegent et al., 1987). This method permitted investigators to expand the assayable target, to allow whole-chromosome painting procedures (Lichter et al., 1988). Chromosome analyses by FISH have led to marked progress in cytogenetics research (reviewed by Trask, 2002). A prime example of the power of hybridization approaches in genome investigation is comparative genomic hybridization, during which deletions and duplications of chromosomal regions are detected by differential fluorescence signals (reviewed by Forozan et al., 1997). However, because the assay does not benefit from preservation of tissue structure or cellular architecture, its future applications are more likely to be in silico than in situ (Lichter et al., 2000). Initially, RNA assays could reliably detect only rather abundant messages, using clone-derived probes (Lawrence and Singer, 1986).

One of the problems with larger probes was that they had to be cut into small pieces of <200 nucleotides (Lawrence and Singer, 1986). If a large probe adhered to the sample non-specifically, it would appear to be a signal, because the great number of fluorochromes would generate an intensely fluorescent spot. This would, for instance, confound detection of genes on chromosomes. Double-stranded DNA probes hybridize within tissues and cells, giving high background. Reduction of probe size led to improved signal-to-noise ratio and thus to single-copy detection of genes on chromosomes (Lawrence et al., 1988). Enhancements in detection and computer processing algorithms have subsequently allowed detection of smaller and smaller targets. Advances in microscope and detector hardware have allowed the low light level produced by FISH to be recorded and analyzed with increasing sensitivity (reviewed by Arndt-Jovin et al., 1985). Mathematical image-processing algorithms have built on this progress to yield super-resolution technology to probe at submicroscopic resolution, using digital image stacks (Carrington et al., 1995). In the trend towards detection of smaller entities, cytogeneticists have focused on analysis of cryptic sub-telomeric karyotypic rearrangements (Brown et al., 2000) and the precise chromosomal mapping of genes (reviewed by Palotie et al., 1996). Those working on mRNA detection can assay single mRNAs and even parts of RNAs (Femino et al., 1998).

New targets led to new applications of the FISH procedure, the popularity of the assay increasing dramatically in the 1990s (Fig. 1). The new avenues of research opened by these applications required that more and more species be simultaneously detected. At first, this was achieved by simultaneous visualization of spectrally distinct fluorophores (Hopman et al., 1986); later, strategies using two principal encoding schemes augmented the approach. First, specific nucleic acid identities can be denoted by binary color combinations, such that each chromosome, gene, or transcript

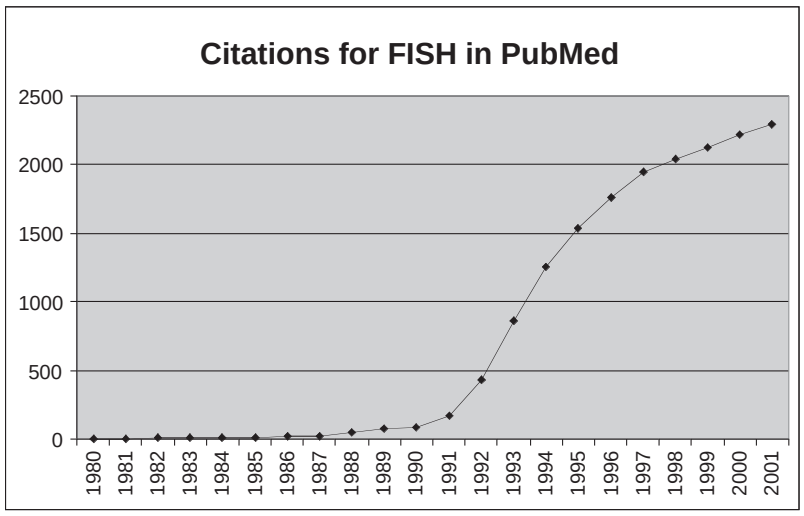

Fig. 1. Number of citations in the PubMed database for the keywords 'fluorescence in situ hybridization' by publication year. Widespread application of the technology was not reached until the introduction of easily accessed methods for probe preparation and detection. After this occurred, the technology spread logarithmically.

is represented by a unique combination of distinct fluorescent signatures. A second scheme employs ratio identity codes that use the same color combination to delineate multiple targets by varying the relative contribution of each color to the total signal. Each of these schemes, as well as the concurrent use of both approaches has raised the number of nucleic acid targets that can be simultaneously detected to dozens (reviewed by Fauth and Speicher, 2001) (Table 1). A major milestone in the detection of chromosome targets was the discrimination of all human chromosomes simultaneously, using computed interpretation of a 5-color scheme (Schrock et al., 1996; Speicher et al., 1996). Although mRNAs can also be visualized in a multiplex fashion (Levsky et al., 2002), FISH analysis of the entire transcriptome in situ is a daunting thought (Fig. 2). One can only speculate that future technologies will feature increasingly higher-order multiplexing, until the number of interesting nucleic acid targets is reached. The technical means for color coding such a large number of entities is already in place (Han et al., 2001), although reduction to practice will be difficult and a means of deciphering spatially overlapping signals will need to be developed.

Quantitation and analysis

Quantitative analysis of fluorescence images was first used as the basis for rudimentary cytogenetic tests (Pinkel et al., 1986), and the use of charge-coupled device digital cameras for the detection of fluorescent signals (reviewed by Tanke et al., 1995) soon allowed the technique to be applied to the analysis of mRNA as well (Pachmann, 1987). Chief concerns in fluorescence assays are the reproducibility and irregularity of the signal and background autofluorescence. Not only do sample-to-sample measurements show variation, but material from the same slide and even the same cells has also been shown to be unevenly fluorescent (Nederlof et al., 1992a). Many schemes have been introduced to compensate for the autofluorescence inherent in certain tissues. In sample preparation, the use of reducing agents including sodium borohydride (Baschong et al., 2001) and pre-treatment by irradiation with light (Neumann and Gabel, 2002) have been shown to reduce non-specific background signal. As these and other similar treatments are not always possible and are seldom completely effective, imaging analysis methods are now under development to subtract autofluorescent signatures from image data mathematically. In general, the total spectral data recorded from a sample consists of true signal and a number of components of noise, the profile for each of which can be measured and removed digitally by procedures such as independent components analysis (Bingham and Hyvarinen, 1997). Given the progressive use of FISH to image many distinct targets at once, images that are composed of more and more spectral components are recorded for data analysis. Such multi-color approaches have their own limitations, including differential intensity of fluorophores and color overlaps. As a result, computational methodologies have been introduced to

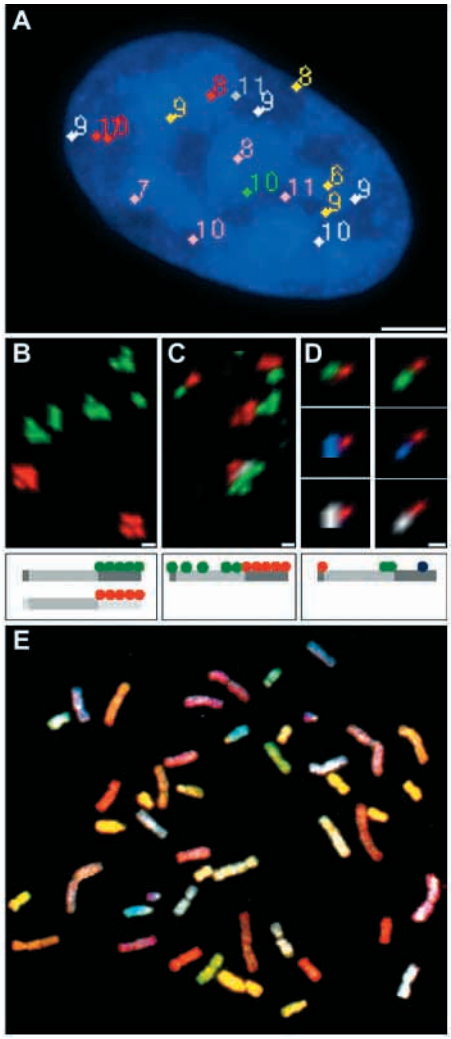

Fig. 2. State of the art in FISH. (A) Many transcription sites (10) detected using barcoded probes can determine the gene expression pattern of each cell (Levsky et al., 2002) (B-D). Detection of single mRNA molecules using double or triple colored probes (Femino et al., 1998). (E) Detection of 24 chromosomes using spectral imaging (Macville et al., 1997).

Table 1. Selected milestones in the development of multi-target FISH

	DNA/gene	mRNA/expression
First in situ detection	Bauman et al., 1980	Singer and Ward, 1982
Two-color detection	Hopman et al., 1986	Dirks et al., 1990
Three-color detection	Nederlof et al., 1989	Dirks et al., 1991
Combinatorial color-coding (M-FISH)	Nederlof et al., 1990	Levsky et al., 2002
Ratio color-coding	Nederlof et al., 1992b	–
Combinations and ratios (COBRA)	Tanke et al., 1999	–

The detection of multiple entities, or multiplexing, is integral to modern FISH approaches. While expression and genotypic analyses have benefited from miniaturization and parallel analysis, in situ techniques have been constrained by detection limitations within the nucleus. This limitation has been overcome, to some extent, by the use of color-coding schemes. The resolution limit of fluorescence microscopy has yet to be approached.

re-balance multi-color images composed of bands of varying strengths and to correct for spectral signal overlaps (Castleman, 1998).

Difficulties inherent in objective analyses of FISH images have impeded but not stopped the development of automated algorithms for interpretation. Detection of DNA targets with large probes and the use of multi-color fluorescent cytometry algorithms (Galbraith et al., 1991) have allowed the production of automated mechanisms for assisting pathologists (Piper et al., 1994). In addition, the use of diagnostic probe sets and dot-counting approaches have yielded independent platforms capable of making simple diagnostic conclusions (Piper et al., 1990). Although methods have been introduced to analyze and optimize these cell classifiers (Castleman, 1985; Castleman and White, 1980; Castleman and White, 1981), manual cytopathology remains the gold standard for reliable tissue analysis, and automated mechanisms that can yield comparable data are still in development. Nevertheless, the benefits of high-throughput analysis of cell preparations, namely objective, computerized interpretation of cell samples on fixed substrates cannot be understated in the future development of diagnostic medicine. Although morphological analyses remain best suited to human operators, the ability to assay many molecular signatures rapidly in cells is only possible through computer-assisted approaches. Automated processing has recently been extended from the detection of specific DNA loci (Lawrence et al., 1988) and sites of transcription (Lawrence et al., 1989) to the determination of functional cell states by multi-gene transcriptional profiling (Levsky et al., 2002). The ability to assess accurately the transcriptional state of individual cells in situ has begun to influence the way we conceptualize single-cell versus tissue-level gene expression as well as study transcription activation, co-expression, and nuclear structure-function (Levsky and Singer, 2003).

Advancing technology

Whereas the initial development of FISH involved expansion of the types of probe and number of detectable targets, the outlook for future development of fluorescence techniques will include expansion of the subjects of investigation. Clinical application of fluorescence imaging will require further advances in mechanization that allow the probes to be delivered, imaged, and analyzed automatically, thereby reducing operator-to-operator variability. Specimen thickness has been a limiting factor in the types of sample that can be analyzed with fluorescence microscopy. Until recently microstructure analysis based on methods such as confocal microscopy and optical coherence tomography was limited to specimens of approximately 1-2 mm thickness. A recent technological improvement known as optical projection tomography has allowed image-reconstruction of specimens up to 15 mm thick, setting the stage for more broad application to biological and clinical specimens (Sharpe et al., 2002).

FISH techniques for detecting RNAs have been introduced to living cells (reviewed by Boulon et al., 2002), using either fluorophores that can be ‘un-caged’ in vivo (Politz and Singer, 1999) or probes that fluoresce only when hybridized (Tyagi and Kramer, 1996). Both of these innovative approaches circumvent the high background usually found in scenarios with unbound probe present (such as living cells), to allow the investigator to follow the creation and travels of mRNAs. These approaches are more easily applied to different target molecules than non-hybridization based GFP-fusion protein systems that bind a unique nucleic acid motif (Bertrand et al., 1998). One drawback of live-cell in situ hybridization as opposed to GFP-based assays is that FISH requires mechanical disturbance of the cell to introduce probes. These techniques allow deeper study of live gene expression in a minimally disturbed context, but must be interpreted with consideration of the possible artifacts that may result as physiological ramifications of hybridization. The separation drawn between approaches using fluorescent proteins and FISH should not be considered absolute. In fact, the compatibility of FISH and technologies employing fluorescent fusion proteins promises to allow simultaneous monitoring of proteins and nucleic acids of interest.

The use of multi-photon approaches will also expand application of fluorescence imaging. In multi-photon microscopy, a laser source fires short bursts of photons that are focused by the microscope to arrive in pairs or triplets such that they summate to excite the fluorophore of interest. Near-infrared excitation light is used, which penetrates biological specimens more deeply and is far less toxic to live samples than visible light. This scheme has already allowed the application of fluorescence imaging to many living systems, including whole animals. Owing to limitations of our ability to introduce synthetic probes into organisms, most current applications of in vivo fluorescence imaging involve naturally occurring fluorescent molecules or bioluminescence (reviewed by Contag and Bachmann, 2002). Native fluorescent signatures that are present in tissues due to normal physiology or pathophysiological processes can encode important clinical information (reviewed by Konig, 2000). Should a form of organism-friendly probe become a reality, the power to discern many specific nucleic acids could be applied to non-invasive diagnostics, providing an informative adjunct to current methods of medical imaging.

Diagnostic FISH

The development of in situ technologies has provided us with a wealth of information regarding the locations and expression patterns of genes in single cells. More complete gene expression profiles of single cells will provide a new level of insight into the correlation of gene expression patterns with particular cellular phenotypes. This will be particularly important in studies of development and disease progression, where complicated, finely demarcated gene expression programs are in play.

Surprises are in store, however. The stochastic nature of gene expression revealed by this kind of approach indicates that perhaps our conception of a precisely regulated gene expression pattern is too constrained. Higher levels of tolerance for diversity in cell expression patterns may require a different model (Levsky and Singer, 2003). Computer-interpreted FISH assays are now sufficiently advanced to provide enormous amounts of data from a single cell, and even more from a tissue section. Measurement of expression from 20 genes by scoring for activity of neither, both, or one of the two alleles as mere binary ‘on or off’ signals, yields 3^{20} or greater than three billion bits of information per cell. If expression information

concerning 100 genes were to be assayed, the information density would increase to 3^{100} bits (on the order of 10^{47}). This exponential increase indicates that high-throughput data processing of gene expression information will have to evolve with the technology. The mere enormity of data may reveal insights not dreamt of in our philosophy.

The ability to visualize RNA movement in living cells will provide models for how and where specific sequences are expressed and the steps by which transcripts are processed and exported from the nucleus. Our understanding of infectious disease will benefit from elucidation of how retroviruses direct nuclear import, trafficking and packaging for export into infectious particles. We are just beginning to understand the mechanisms by which specific RNAs are localized to subcellular regions of oocytes and some somatic cells for the purposes of asymmetric translation and how this is used to effect permanent structural changes – for instance, in synaptogenesis. When the tools become available for us to visualize multiple gene expression patterns in living cells, we will finally be able to fulfill the promise of FISH technology by building and testing models of molecular transcriptional dynamics within the true native context of the cell.

The traditional route to diagnosis has been through morphological evaluation of biopsy specimens and the correlation of this analysis with clinical outcomes. The morphological basis of diagnosis has its limitations. It is well known that tumors that look alike under the microscope, and that appear phenotypically similar, may have radically different clinical courses in real patients. The new field of molecular pathology attempts to obviate the ambiguities of morphology by studying the origins of disease through characterization of genetic mutations and gene products. These investigations promise to provide more reliable biomarker information, founded on recent bioinformatics advances made possible by expression studies using micoarrays and serial analysis of gene expression (SAGE). Transcriptional alterations associated with malignant transformation and markers that correlate with cancer progression are being identified. The mechanism by which these data can be incorporated in the pathologist's 'tool box' is currently being developed.

The recently developed tissue microarray technology is an ideal platform for the introduction of high-throughput molecular profiling of tumor specimens at the single cell level. To construct a tissue microarray, small core biopsies are taken from representative areas of paraffin-embedded tumor tissues and assembled in a single block. Microtome sections are taken from the tissue microarray and placed on glass slides for rapid and efficient molecular analyses. In addition to pathological specimens such as tumor tissues, microarrays generally contain corresponding normal tissue and internal controls. The entire group of samples is analyzed simultaneously in one experiment, providing enormous amounts of correlative information about specific biomarkers, in the context of rigorous procedural controls. The next challenge will be to apply multi-gene FISH technology to these samples to correlate putative genes of prognostic value with specific morphological features initially, and then extend studies to samples where the morphology is not sufficiently informative. Certain genes can then be associated with the pre-cancerous state, for instance. Through such developments, one can foresee how molecular pathology could eventually surpass the limitations of morphological pathology. This would allow more judicious use of minimally invasive biopsy techniques that sacrifice retrieved tissue morphology in favor of comfort of the patient. FISH has already colored the way that we visualize and conceptualize genes, chromosomes, transcription and nucleic acid movements. What remains to be seen is how exhaustive molecular analysis of single cells and tissue samples will impact how we identify, diagnose and alter the course of genetic pathology. Over the long term, it is expected that databases correlating gene expression patterns on the single cell level will accumulate as investigators and industries employ the technology of FISH with their favored biomarkers. Ultimately, FISH will be the preferred approach to anticipate the complicated components of gene expression leading to disease.

Our work is supported by the Innovative Molecular Analysis Technologies program at the National Cancer Institute, and research and training grants from the NIH.

References

Arndt-Jovin, D. J., Robert-Nicoud, M., Kaufman, S. J. and Jovin, T. M. (1985). Fluorescence digital imaging microscopy in cell biology. *Science* **230**, 247-256.

Baschong, W., Suetterlin, R. and Laeng, R. H. (2001). Control of autofluorescence of archival formaldehyde-fixed, paraffin-embedded tissue in confocal laser scanning microscopy (CLSM). *J. Histochem. Cytochem.* **49**, 1565-1572.

Bauman, J. G., Wiegant, J., Borst, P. and van Duijn, P. (1980). A new method for fluorescence microscopical localization of specific DNA sequences by in situ hybridization of fluorochromelabelled RNA. *Exp. Cell Res.* **128**, 485-490.

Bertrand, E., Chartrand, P., Schaefer, M., Shenoy, S. M., Singer, R. H. and Long, R. M. (1998). Localization of ASH1 mRNA particles in living yeast. *Mol. Cell* **2**, 437-445.

Bingham, E. and Hyvarinen, A. (1997). A fast fixed-point algorithm for independent component analysis. *Int. J. Neural Syst.* **10**, 1-8.

Boulon, S., Basyuk, E., Blanchard, J. M., Bertrand, E. and Verheggen, C. (2002). Intra-nuclear RNA trafficking: insights from live cell imaging. *Biochimie* **84**, 805-813.

Brown, J., Horsley, S. W., Jung, C., Saracoglu, K., Janssen, B., Brough, M., Daschner, M., Beedgen, B., Kerkhoffs, G., Eils, R. et al. (2000). Identification of a subtle t(16;19)(p13.3;p13.3) in an infant with multiple congenital abnormalities using a 12-colour multiplex FISH telomere assay, M-TEL. *Eur. J. Hum. Genet.* **8**, 903-910.

Carrington, W. A., Lynch, R. M., Moore, E. D., Isenberg, G., Fogarty, K. E. and Fay, F. S. (1995). Superresolution three-dimensional images of fluorescence in cells with minimal light exposure. *Science* **268**, 1483-1487.

Castleman, K. R. (1985). Estimation of sampling errors. *Cytometry* **6**, 276-277.

Castleman, K. R. (1998). Concepts in imaging and microscopy: color image processing for microscopy. *Biol. Bull.* **194**, 100-107.

Castleman, K. R. and White, B. S. (1980). Optimizing cervical cell classifiers. *Anal. Quant. Cytol.* **2**, 117-122.

Castleman, K. R. and White, B. S. (1981). The effect of abnormal cell proportion on specimen classifier performance. *Cytometry* **2**, 155-158.

Contag, C. H. and Bachmann, M. H. (2002). Advances in in vivo bioluminescence imaging of gene expression. *Annu. Rev. Biomed. Eng.* **4**, 235-260.

Dirks, R. W., van Gijlswijk, R. P., Tullis, R. H., Smit, A. B., van Minnen, J., van der Ploeg, M. and Raap, A. K. (1990). Simultaneous detection of different mRNA sequences coding for neuropeptide hormones by double in situ hybridization using FITC- and biotin-labeled oligonucleotides. *J. Histochem. Cytochem.* **38**, 467-473.

Dirks, R. W., van Gijlswijk, R. P., Vooijs, M. A., Smit, A. B., Bogerd, J., van Minnen, J., Raap, A. K. and van der Ploeg, M. (1991). 3′-end fluorochromized and haptenized oligonucleotides as in situ hybridization probes for multiple, simultaneous RNA detection. *Exp. Cell Res.* **194**, 310-315.

Fauth, C. and Speicher, M. R. (2001). Classifying by colors: FISH-based genome analysis. *Cytogenet. Cell Genet.* **93**, 1-10.

Femino, A. M., Fay, F. S., Fogarty, K. and Singer, R. H. (1998). Visualization of single RNA transcripts in situ. *Science* **280**, 585-590.

Forozan, F., Karhu, R., Kononen, J., Kallioniemi, A. and Kallioniemi, O. P. (1997). Genome screening by comparative genomic hybridization. *Trends Genet.* **13**, 405-409.

Galbraith, W., Wagner, M. C., Chao, J., Abaza, M., Ernst, L. A., Nederlof, M. A., Hartsock, R. J., Taylor, D. L. and Waggoner, A. S. (1991). Imaging cytometry by multiparameter fluorescence. *Cytometry* **12**, 579-596.

Gall, J. G. and Pardue, M. L. (1969). Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc. Natl. Acad. Sci. USA* **63**, 378-383.

Han, M., Gao, X., Su, J. Z. and Nie, S. (2001). Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. *Nat. Biotechnol.* **19**, 631-635.

Hopman, A. H., Wiegant, J., Raap, A. K., Landegent, J. E., van der Ploeg, M. and van Duijn, P. (1986). Bi-color detection of two target DNAs by non-radioactive in situ hybridization. *Histochemistry* **85**, 1-4.

Hougaard, D. M., Hansen, H. and Larsson, L. I. (1997). Non-radioactive in situ hybridization for mRNA with emphasis on the use of oligodeoxynucleotide probes. *Histochem. Cell Biol.* **108**, 335-344.

Kislauskis, E. H., Li, Z., Singer, R. H. and Taneja, K. L. (1993). Isoform-specific 3′-untranslated sequences sort alpha-cardiac and beta-cytoplasmic actin messenger RNAs to different cytoplasmic compartments. *J. Cell Biol.* **123**, 165-172.

Konig, K. (2000). Multiphoton microscopy in life sciences. *J. Microsc.* **200**, 83-104.

Landegent, J. E., Jansen in de Wal, N., Dirks, R. W., Baao, F. and van der Ploeg, M. (1987). Use of whole cosmid cloned genomic sequences for chromosomal localization by non-radioactive in situ hybridization. *Hum. Genet.* **77**, 366-370.

Langer, P. R., Waldrop, A. A. and Ward, D. C. (1981). Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. *Proc. Natl. Acad. Sci. USA* **78**, 6633-6637.

Lawrence, J. B. and Singer, R. H. (1986). Intracellular localization of messenger RNAs for cytoskeletal proteins. *Cell* **45**, 407-415.

Lawrence, J. B., Singer, R. H., Villnave, C. A., Stein, J. L. and Stein, G. S. (1988). Intracellular distribution of histone mRNAs in human fibroblasts studied by in situ hybridization. *Proc. Natl. Acad. Sci. USA* **85**, 463-467.

Lawrence, J. B., Singer, R. H. and Marselle, L. M. (1989). Highly localized tracks of specific transcripts within interphase nuclei visualized by in situ hybridization. *Cell* **57**, 493-502.

Levsky, J. M. and Singer, R. H. (2003). Gene expression and the myth of the average cell. *Trends Cell Biol.* **13**, 4-6.

Levsky, J. M., Shenoy, S. M., Pezo, R. C. and Singer, R. H. (2002). Single-cell gene expression profiling. *Science* **297**, 836-840.

Levsky, J. M., Braut, S. A. and Singer, R. H. (2003). Single cell gene expression profiling by multiplexed expression fluorescence in situ hybridization: application to the analysis of cultured cells. In *Cell Biology: A Laboratory Handbook* (in press) (ed. J. E. Celis): Academic Press.

Lichter, P., Cremer, T., Borden, J., Manuelidis, L. and Ward, D. C. (1988). Delineation of individual human chromosomes in metaphase and interphase cells by in situ suppression hybridization using recombinant DNA libraries. *Hum. Genet.* **80**, 224-234.

Lichter, P., Joos, S., Bentz, M. and Lampel, S. (2000). Comparative genomic hybridization: uses and limitations. *Semin. Hematol.* **37**, 348-357.

Macville, M., Veldman, T., Padilla-Nash, H., Wangsa, D., O'Brien, P., Schrock, E. and Ried, T. (1997). Spectral karyotyping, a 24-colour FISH technique for the identification of chromosomal rearrangements. *Histochem. Cell Biol.* **108**, 299-305.

Manuelidis, L., Langer-Safer, P. R. and Ward, D. C. (1982). High-resolution mapping of satellite DNA using biotin-labeled DNA probes. *J. Cell Biol.* **95**, 619-625.

Nederlof, P. M., Robinson, D., Abuknesha, R., Wiegant, J., Hopman, A. H., Tanke, H. J. and Raap, A. K. (1989). Three-color fluorescence in situ hybridization for the simultaneous detection of multiple nucleic acid sequences. *Cytometry* **10**, 20-27.

Nederlof, P. M., van der Flier, S., Wiegant, J., Raap, A. K., Tanke, H. J., Ploem, J. S. and van der Ploeg, M. (1990). Multiple fluorescence in situ hybridization. *Cytometry* **11**, 126-131.

Nederlof, P. M., van der Flier, S., Raap, A. K. and Tanke, H. J. (1992a). Quantification of inter- and intra-nuclear variation of fluorescence in situ hybridization signals. *Cytometry* **13**, 831-838.

Nederlof, P. M., van der Flier, S., Vrolijk, J., Tanke, H. J. and Raap, A. K. (1992b). Fluorescence ratio measurements of double-labeled probes for multiple in situ hybridization by digital imaging microscopy. *Cytometry* **13**, 839-845.

Neumann, M. and Gabel, D. (2002). Simple method for reduction of autofluorescence in fluorescence microscopy. *J. Histochem. Cytochem.* **50**, 437-439.

Pachmann, K. (1987). In situ hybridization with fluorochrome-labeled cloned DNA for quantitative determination of the homologous mRNA in individual cells. *J. Mol. Cell Immunol.* **3**, 13-19.

Palotie, A., Heiskanen, M., Laan, M. and Horelli-Kuitunen, N. (1996). High-resolution fluorescence in situ hybridization: a new approach in genome mapping. *Ann. Med.* **28**, 101-106.

Pinkel, D., Straume, T. and Gray, J. W. (1986). Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. *Proc. Natl. Acad. Sci. USA* **83**, 2934-2938.

Piper, J., Fantes, J., Gosden, J. and Ji, L. A. (1990). Automatic detection of fragile X chromosomes using an X centromere probe. *Cytometry* **11**, 73-79.

Piper, J., Poggensee, M., Hill, W., Jensen, R., Ji, L., Poole, I., Stark, M. and Sudar, D. (1994). Automatic fluorescence metaphase finder speeds translocation scoring in FISH painted chromosomes. *Cytometry* **16**, 7-16.

Politz, J. C. and Singer, R. H. (1999). In situ reverse transcription for detection of hybridization between oligonucleotides and their intracellular targets. *Methods* **18**, 281-285.

Puvion-Dutilleul, F. and Puvion, E. (1996). Non-isotopic electron microscope in situ hybridization for studying the functional sub-compartmentalization of the cell nucleus. *Histochem. Cell Biol.* **106**, 59-78.

Raap, A. K. (1998). Advances in fluorescence in situ hybridization. *Mutat. Res.* **400**, 287-298.

Rudkin, G. T. and Stollar, B. D. (1977). High resolution detection of DNA-RNA hybrids in situ by indirect immunofluorescence. *Nature* **265**, 472-473.

Schrock, E., du Manoir, S., Veldman, T., Schoell, B., Wienberg, J., Ferguson-Smith, M. A., Ning, Y., Ledbetter, D. H., Bar-Am, I., Soenksen, D. et al. (1996). Multicolor spectral karyotyping of human chromosomes. *Science* **273**, 494-497.

Sharpe, J., Ahlgren, U., Perry, P., Hill, B., Ross, A., Hecksher-Sorensen, J., Baldock, R. and Davidson, D. (2002). Optical projection tomography as a tool for 3D microscopy and gene expression studies. *Science* **296**, 541-545.

Singer, R. H. and Ward, D. C. (1982). Actin gene expression visualized in chicken muscle tissue culture by using in situ hybridization with a biotinated nucleotide analog. *Proc. Natl. Acad. Sci. USA* **79**, 7331-7335.

Speicher, M. R., Gwyn Ballard, S. and Ward, D. C. (1996). Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nat. Genet.* **12**, 368-375.

Tanke, H. J., Florijn, R. J., Wiegant, J., Raap, A. K. and Vrolijk, J. (1995). CCD microscopy and image analysis of cells and chromosomes stained by fluorescence in situ hybridization. *Histochem. J.* **27**, 4-14.

Tanke, H. J., Wiegant, J., van Gijlswijk, R. P., Bezrookove, V., Pattenier, H., Heetebrij, R. J., Talman, E. G., Raap, A. K. and Vrolijk, J. (1999). New strategy for multi-colour fluorescence in situ hybridisation: COBRA: COmbined Binary RAtio labelling. *Eur. J. Hum. Genet.* **7**, 2-11.

Trask, B. J. (2002). Human Genetics and Disease: Human cytogenetics: 46 chromosomes, 46 years and counting. *Nat. Rev. Genet.* **3**, 769-778.

Tyagi, S. and Kramer, F. R. (1996). Molecular beacons: probes that fluoresce upon hybridization. *Nat. Biotechnol.* **14**, 303-308.

van der Ploeg, M. (2000). Cytochemical nucleic acid research during the twentieth century. *Eur. J. Histochem.* **44**, 7-42.

Wiegant, J., Ried, T., Nederlof, P. M., van der Ploeg, M., Tanke, H. J. and Raap, A. K. (1991). In situ hybridization with fluoresceinated DNA. *Nucleic Acids. Res.* **19**, 3237-3241.