Sensitive detection of RNAs in single cells by flow cytometry

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ABSTRACT

A rapid and sensitive fluorescent *in situ* hybridization method has been developed to probe RNA contents of individual cells by flow cytometry. Fixed cells in suspension were hybridized with 5' end-fluorophore-labeled oligodeoxynucleotides complementary to defined regions of the RNA of interest and analyzed by flow cytometry. With this method, we monitored combinations of histone H4 mRNA, 18S rRNA and 28S rRNA levels in synchronized HeLa S3 cells by multi-color analysis. A fluorescence signal equivalent to 1800 copies of histone H4 mRNA per cell was detected with signal-to-background ratio of 5.4. If non-specific binding of the fluorophore-labeled probe can be reduced, as few as 100 copies of mRNA of the size of H4 could be detected in individual cells by flow cytometry.

INTRODUCTION

In the past several years, fluorescent *in situ* hybridization (FISH) has become an increasingly important tool for both mapping genes to specific regions on chromosomes (1–7) and detecting specific RNA or DNA sequences in isolated cells and tissue sections (8–15). Fluorescent probes are not only stable and safe to use, but also permit rapid detection and relatively high resolution in comparison with the radioactive methods. In comparison with other formats of nucleic acid hybridization, FISH offers several advantages. First, it allows for the analysis of gene expression or virus infection in heterogeneous cell populations where only one population of the cells is expressing the gene of interest. Second, it allows for the simultaneous detection of multiple substances within individual cells. In addition, the sensitivity of FISH is now approaching that of radioisotope-based methods (16). These properties of FISH make it not only an important investigative but also a potential diagnostic technique. Instrumentation capable of detection of fluorescent hybridization probes has also evolved. Modern electronic imaging microscopes allow sensitive detection and localization of multiple fluorescence signals within individual cells (17) and flow cytometry permits the measurement of individual cell fluorescence with high

accuracy and speed. Large cell populations can be statistically characterized and rare cells detected (18). While multicolor flow cytometry has been particularly useful in lymphocyte immunophenotyping, only a few studies have targeted the detection of specific RNA or DNA sequences by flow cytometry (11–15).

In the present study, we established a rapid flow cytometric FISH method. With this method, we were able to monitor histone gene expression during HeLa S3 cell cycle and to simultaneously detect three RNA sequences in single cells. Quantification of RNA sequence per cell by flow cytometry was also examined.

MATERIALS AND METHODS

Cell culture and synchronization

HeLa S3 cells obtained from ATCC were maintained in 150×20 mm petri dishes in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum. Cells were passed twice a week at a density of 2.5×10^5 cells per dish. HeLa S3 cells were synchronized by means of subsequent thymidine and aphidicolin block described previously (19). Briefly, 3 days after plating, cells were treated with MEM containing 2 mM thymidine for 12 hours, then the cells were washed with thymidine-free medium three times and released into MEM containing 2.4×10^{-5} M thymidine and deoxycytidine for 9 hours. The cells were then blocked again for 12 hours by adding aphidicolin to a final concentration of 5 µg/ml medium. Upon release into thymidine-free medium, cells progressed into S phase, G2 phase and mitosis. Cells were harvested by trypsin treatment. Typically, 4×10^6 cells were obtained per dish.

Cell fixation

Cells for *in situ* hybridization were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) containing 5 mM MgCl₂. Fixation was done at room temperature for 15 minutes. After fixation, the cells were washed twice with PBS and stored in PBS in refrigerator up to 2 weeks. Cells for propidium iodide staining of DNA content were fixed with 70% ethanol at room temperature and vigorously vortexed for 4 minutes and placed in refrigerator for at least one hour before staining.

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Oligonucleotide synthesis and purification

Nine 25-base oligonucleotide probes complementary to 70% of the histone H4 coding region (there is a 10-base-space between every two probes along the sequence) were synthesized at 1.3 μ mol scale on the Gene Assembler Plus (Pharmacia LKB Biotechnology). The DNA synthesis chemicals were obtained from Pharmacia LKB Biotechnology and the new PAC phosphoramidites were used. At the end of the synthesis, an automated 5'-aminoalkylation (Pharmacia LKB) was performed. In the first step, the 5'-hydroxyl group of the oligonucleotide was activated by carbonyldiimidazole (CDI). The resulting 5'-imidazolyl derivative was then reacted with diaminopropane giving a 5'-aminoalkylated oligonucleotide with the diamine attached by means of a carbamate linkage. After deprotection with concentrated ammonium hydroxide at room temperature overnight, the probes were purified using NAP 25 (Pharmacia LKB) and Biogel P4 columns subsequently. Similarly, two 25-base-long probes were made to detect 18S and 28S ribosomal RNAs. The sequences are TCACCTCTAGCGGCGCAATACG-AAT for 18S rRNA and ACCTTTTCTGGGGTCTGATG-AGCGT for 28S rRNA.

Synthesis of fluorophore-conjugated oligonucleotides

The nine H4 probes were labeled with Cy5.18 and the 18S and 28S rRNA probes were labeled with Cy3.18 and OCy3 respectively. Typically, 60nmol of each oligonucleotide was labeled. The pH of the oligonucleotide solution was adjusted to 9.5 with Na_2CO_3 , then 300 nmol ($5\times$ excess) dye dissolved in dry DMF was added. The reaction mixture was vortexed and incubated in dark for 2 hours. To remove unreacted dye, the labeling mixture was applied to Biogel-P4. The unlabeled oligonucleotides were removed by reverse phase HPLC on a 150×4.1 mm Hamilton PRP-1 column using a biphasic linear gradient previously described (20). The purified probes were concentrated down and dialyzed against double distilled water and stored at -20°C .

In situ hybridization

1 μ l of freshly made 10% diethylpyrocarbonate (DEPC) in 70% ethanol was added to every 50 μ l of fixed cell suspension in PBS (typically, each cell preparation contains 10^6 cells) and let stand at room temperature for 15 min. The cells were spun down and then were suspended in 20 mM Tris-HCl containing 2 mM CaCl_2 , pH7.5 and 1 mg/ml proteinase K and incubated at 37°C for 7.5 min. After two washes with Hank's balanced salt solution (HBSS), the cells were spun down and suspended in HBSS. One volume of $20\times\text{SSC}$ and 2 volumes of formamide (Fisher, molecular biology grade) were added to the cell suspension. Meanwhile, the appropriate amount of dried probe was dissolved in 15 μ l hybridization buffer ($5\times\text{SSC}$, 50% formamide, 500 mg/ml E. coli tRNA, 100 mg/ml sheared salmon sperm DNA and 0.5% SDS), heated at 100°C for 2 min and cooled down on ice. The cells were suspended by repeated pipetting in 15 μ l of the probe in hybridization buffer. Hybridization was carried out at 40°C with gentle shaking for 4 hours. After hybridization, 300 μ l of hybridization buffer without the probes was added to the cells and was further incubated for 45 min to remove mismatched probes. The cells were then pelleted and washed successively with $2\times\text{SSC}$ and $0.1\times\text{SSC}$ for 30 min each to remove non-specific binding. Finally, the cells were suspended in 1 ml HBSS for flow experiments.

RNA extraction and RNA blot analysis

The total cytoplasmic RNA was extracted from G1/S boundary cells or S phase cells and was separated by electrophoresis on formaldehyde-containing 1% agarose gels and blotted onto Nytran filter following the protocol described by Maniatis et al. (21). The blot was prehybridized in $5\times\text{SSPE}$, $10\times\text{Denhardt's}$, 100mg/ml denatured DNA, 0.4% SDS for 2 hours at 42°C . The 5'-end fluorophore-labeled probes were labeled with ^{32}P using a 3'-end labeling system (Promega) and $[\alpha\text{-}^{32}\text{P}]$ ddATP (Amersham). The 50 μ l labeling mixture contained 500 mM cacodylate buffer pH6.8, 5 μ l $[\alpha\text{-}^{32}\text{P}]$ ddATP (5000ci/mmol, 10mCi/ml), 10–20 units of terminal deoxynucleotidyl transferase and 1.1 pmoles of each of the nine oligo probes. Then the nine ^{32}P -labeled oligo probes were added and hybridization was carried out overnight at 42°C .

Propidium iodide staining

Cells fixed in 70% ethanol were washed twice with sodium citrate buffer (50 mM sodium citrate pH7.8, 0.15 mM NaCl and 0.5% triton X-100). PI staining solution (50 μ g propidium iodide and 50 mg RNase A per ml citrate buffer) was added to the cell pellet, mixed well and stained at 4°C overnight. After staining, the cells were stored in isotonic solution (50 mg propidium iodide per ml citrate buffer) until flow.

Flow cytometric analysis

Flow cytometry measurements were made using a dual laser FAC-440 equipped with a Consort 40 collection system. The Argon laser was set to 400 mw at 488 nm and the Krypton laser was set to 200 mw at 647 nm. Cy5 information was acquired using the light selection filter of 670/13.5 nm (central wavelength/full

Fig. 1. Optimization of the hybridization parameters. HeLa S3 cells harvested at mid-S phase were fixed and hybridized with the H4 probes for 4 hours (A) at various temperatures. (B) at 40°C with various amounts of the H4 oligo probes, from 0.05 picomole to 1 picomole of each of the nine oligos per million cells. The Cy5.18 fluorescence intensity was collected on linear scale.

bandwidth at half-maximal transmission). OCy3, Cy3 and PI were excited utilizing the 488 nm Argon line and the fluorescence information was obtained using the filters of 530/30 nm (OCy3) and 575/26 nm (Cy3 and PI). Because of the bleed over of fluorescence from the OCy3 into the Cy3 channel, compensation was accomplished by manually adjusting the compensation control using a sample containing OCy3 positive and negative control cells until both populations were horizontal to each other.

Flow cytometric calibration curve

Polystyrene latex particles (2.54 microns) coated with avidin (a generous gift from Dr. Jeff Wang, Pandex division of Baxter Healthcare Corporation) were used as standards. Mono-functional Cy5 (Mujumdar et al., unpublished result) was reacted with biocytin to obtain biotin-Cy5 adduct. Aliquots of 1 million beads were incubated with various defined amounts of biotin-Cy5 adduct (0 to 360,000 adducts per bead) in 0.1 ml PBS for 30 minutes. These standard bead samples were diluted to 1 ml prior to flow cytometric analysis and can be stored up to two months in refrigerator.

RESULTS AND DISCUSSION

Optimization of *in situ* hybridization

The hybridization procedure was based on previous reports with some modifications (11, 22). Some hybridization parameters were optimized with our probes in order to obtain the best signal-to-background ratio as measured by flow cytometry. For example, 4% paraformaldehyde was chosen as the fixative in our protocol, since the precipitating fixatives we tested (ethanol:HAC 3:1 and methanol:acetone 1:1) were not sufficient for cellular RNA retention and gave much less signal (data not shown). Glutaraldehyde was avoided because it caused high autofluorescence. We found it important to determine the optimal hybridization temperature experimentally. The optimal hybridization temperature was 40°C for the H4 probes as shown in Fig. 1A. At lower temperatures, non-specific binding became greater, reducing the signal-to-background ratio. Higher temperatures decreased the stability of the hybrids, therefore also

lowering signal-to-background ratio. It is also essential to optimize the probe-to-target ratio, since addition of probe in excess of the amount causing signal saturation leads to increased non-specific binding. As shown in Fig. 1B, saturation point was reached at 0.4 picomole of each of the nine H4 probes per million cells. Therefore, 0.5 picomoles per million cells was routinely used as probe-to-target ratio. For each of the ribosomal RNA probes, saturation was reached at 15 picomole of the probe per million cells (data not shown) which was routinely used.

Probe specificity

The specificity of the nine histone H4 oligonucleotide probes was tested by both RNA blot analysis and flow cytometric analysis. Because of the aminolinker at the 5'-end of the nine H4 oligo probes, 3'-end labeling with [α - 32 P]ddATP catalyzed by terminal transferase was performed. The RNA blot (Fig. 2A) clearly shows that the probes bound to a S-phase specific 340-nucleotide message. Only very slight cross-hybridization to the 18S rRNA was observed.

Flow cytometry experiments also confirmed the specificity of the H4 probes. It is well-known that histone mRNA accumulates during S phase; G1, G2, and M phase cells have, at most basal level of histone messages. As shown in Fig. 2B, after hybridization with the H4 probes, the fluorescence of hybridized cells at mid-S phase (5 hours after release into S phase) is 53 channels higher on a log scale than that of the G2/M phase cells (12 hours after release into S phase). This difference corresponds to a factor of 8 in cell fluorescence. The signal was not only S phase-specific but also RNA specific, since RNase-treated S phase cells showed fluorescence 50 channels lower on a log scale than that of non-treated S phase cells (Fig. 2B). A typical signal-to-background ratio is 5.4 which is defined as F/F_c , where F is linear fluorescent intensity of the positive cells and F_c is the linear fluorescent intensity of the negative control cells.

Cell cycle-dependent histone gene expression

In order to demonstrate the utility of this simple approach of flow cytometric analysis of mRNA levels, we chose to examine histone H4 gene expression and its relationship with DNA synthesis by

Fig. 2. Probe specificity tested by (A) RNA blot analysis. HeLa S3 cells were synchronized at G1/S boundary by thymidine-aphidicolin block (22). The total cytoplasmic RNA was extracted from G1/S boundary cells (lane 1) and from mid-S phase cells (lane 2) and resolved on formaldehyde-containing 1% agarose gel. The blot was hybridized with the 3' end 32 P-labeled H4 probes. (B) flow cytometric analysis. HeLa S3 cells were harvested 5 hours and 12 hours after released into S phase and hybridized *in situ* with the nine Cy5.18-labeled H4 probes. Cy5.18 fluorescence information was acquired on log scale. a) unstained cells. b) cells harvested 5 hours into S phase and treated with RNase prior to the hybridization. c) cells harvested 12 hours after release and hybridized with the probes. d) cells harvested 5 hours into S phase and hybridized with the probes.

flow cytometric FISH. This is an excellent test system, because it has been well documented that, in most eukaryotic cells, histone gene expression is temporally and functionally coupled to DNA synthesis, occurring predominantly during the S phase of the cell cycle. Many researchers have explored the regulation of histone gene expression and DNA synthesis during the HeLa cell cycle with radioisotopic methods (19, 23–28) and have demonstrated that there is a large increase in the levels of histone mRNAs in the S phase of the cell cycle.

HeLa S3 cells were synchronized at the G1/S boundary by thymidine aphidicolin block as described in Materials and Methods. After release into drug-free medium, cells were harvested at hourly interval for 12 hours. We divided the cells

into two portions, measuring H4 mRNA on one sample and DNA content on the other. We used a cocktail of the nine Cy5.18-labeled H4 oligonucleotide probes for measurement of histone mRNA by *in situ* hybridization. Propidium iodide (PI) staining was used to quantify the DNA content. Examples of flow cytometric histograms are shown in Fig. 3B. The G1/S boundary cells show basal level of Cy5 fluorescent intensity. At this time the PI fluorescence indicates the single complement of DNA in cells. Upon release, the Cy5 and PI fluorescent signals immediately increase. The histone signal (Cy5) reaches the maximum at the 5th hour after release and by the 7th hour most of the cells have lost their H4 mRNA. The DNA content increases until the 6th hour at which it has doubled. Between the 9th and the 10th hour, the cells divide. At the 12th hour the cells have low H4 mRNA content and a single complement of DNA. Fig. 3A summarizes the time course study of H4 and DNA synthesis as a synchronized HeLa S3 cell population entered into S phase. It is clear from our result that histone mRNA is accumulated in S phase while DNA synthesis is in progress. Once DNA synthesis is accomplished, histone H4 mRNA quickly decreases to a background level.

Quantification of mRNA in single cells by flow cytometric FISH

To estimate the sensitivity of the method, we determined the minimum number of copies of RNA molecules per cell that can be detected by flow cytometry using end-labeled fluorescent oligonucleotides. We approached this question by using a series of fluorescent particles labeled with different known amounts of Cy5 as flow cytometer calibration standards. Avidin-coated latex microspheres were labeled with known numbers of biotinylated Cy5 fluorophores. Plots of numbers of fluorophores per particle versus mean linear channel number showed a linear relationship with a sensitivity of 15,500 Cy5 equivalents per linear fluorescence unit and a linear-correlation coefficient of 0.999 (Fig. 4). The saturation occurred at about 360,000 dyes per particle. All calibration experiments were performed in a linear labeling range of 0–60,000 dyes per particle. The quantum yield of Cy5 labeled on the calibration particles has been assumed to be the same as that of Cy5 labeled on the H4 oligonucleotide probe in the cell. Since Cy5 is relatively insensitive to the environment, this should be a valid assumption.

Fig. 3. Histone H4 gene expression and DNA synthesis during HeLa S3 cell cycle monitored by flow cytometry. HeLa S3 cells were synchronized at G1/S boundary by thymidine-aphidicolin block (22). The cells were harvested at hourly interval after release into S phase. The cells were split into two portions, one for *in situ* hybridization with H4 probes and the other for PI staining of DNA content. (A) Time course of H4 mRNA accumulation (●) and DNA content (○). (B) flow cytometric histograms of H4 mRNA signal (left panel) and DNA content (right panel) of cells at 0, 5, 7, and 12 hours after released into S phase.

Fig. 4. Cy5 channel calibration. Polystyrene latex particles (2.54 microns) coated with avidin were used as standard. Aliquots of 1 million beads were incubated with various amounts of biotin-Cy5.18 adduct (0 to 60,000 adducts/bead) in 0.1 ml PBS for 30 minutes. These standard bead samples were diluted to 1 ml prior to analysis by flow cytometry. Cy5 fluorescence was collected on linear scale at 900 volts.

When the calibration particles were analyzed on a flow cytometer under the same conditions for which the cell samples were run, the number of dye molecules per cell can be estimated. Table 1 shows the relative linear fluorescence and the number of Cy5 equivalents per cell. The negative control cells (12 hour cells and RNase-treated 5 hour cells) had an average background labeling of about 5,000 Cy5 equivalents per cell. Cells 5 hours into S phase had about 21,000 Cy5 molecules per cell. The difference between the number of Cy5 equivalents per S phase cell and per negative control cell ($\Delta = 16,000$) divided by 9 (the number of Cy5 reporter groups decorating each H4 message) gives a minimum estimate of 1800 histone H4 mRNA molecules per cell. This minimum assumes that the hybridization efficiency was 100% for all of the nine probes and that no message was lost during the fixation and hybridization.

We believe that non-specific binding (NSB) of Cy5-labeled probe to non RNA structures within cells is the major limiting factor in this technique. Cell autofluorescence and instrumentation noise at the long wavelength of Cy5 excitation (647nm) corresponds to only about 950 Cy5 dye equivalents or about 100 histone H4 mRNA molecules. However, fluorescence from Cy5-labeled H4 probe bound to RNase treated cells corresponds to approximately 4000 Cy5 equivalents or about 450 histone H4

Table 1. Quantification of Cy5.18 fluorescence in cells.

samples	linear fluorescent intensity	number of Cy5 equivalents per cell
unstained cells	0.218	950
0 hour cells	2.36	4,800
12 hour cells	1.98	3,500
5 hr RNase-treated cells	2.59	6,500
5 hour cells	12.56	21,000

After hybridization with the H4 probes, unstained, negative control and positive cells were analyzed by flow cytometry under the exact same conditions as that used for running calibration particles. Number of Cy5 equivalents per cell in each sample was shown.

Table 2. Sensitivity comparison of fluorophore direct labeled- and biotinylated oligo probes in FISH.

28S rRNA probe	secondary reagent	linear fluorescent intensity		signal-to-background
		RNase-treated	non-treated	
Cy5 conjugate	none	4.43	117.45	26.5
Biotin conjugate	Cy5-avidin	12.98	208.98	16.1
	Cy5-streptavidin	13.45	343.27	25.5
FITC conjugate	none	3.70	44.16	11.9
Biotin conjugate	FITC-avidin	7.91	21.99	2.78
	FITC-streptavidin	11.58	35.19	3.04

Cycling HeLa S3 cells were harvested, fixed in 4% paraformaldehyde, and hybridized with Cy5 labeled-, FITC labeled- or biotinylated 28S rRNA probe. Negative control cells were treated with RNase prior to the hybridization. After the post-hybridization washes, while the cells hybridized with the directly labeled probes were ready for flow experiment, the cells hybridized with the biotinylated probe were blocked with 4×SSC, 1% Tween-20, 1% BSA for 10 min at 37°C. The cells then were incubated in the solution containing 5 mg/ml Cy5-avidin, Cy5-streptavidin, FITC-avidin or FITC-streptavidin for 30 min at 37°C, followed by three washes with 4×SSC, 1% Tween-20 at room temperature.

mRNA molecules. Further experiments are needed to determine whether the NSB is caused by the Cy5 label or by sticking of the oligonucleotide. If NSB can be significantly reduced, as low as 100 RNA molecules could be detected if each RNA molecule had 10 Cy5 reporter groups. Additionally, attachment of more and brighter fluorochromes to each oligonucleotide may improve signal-to-background substantially provided that NSB is not also enhanced. However, in the shorter wavelength range, cell autofluorescence limits the sensitivity of the method.

Comparison of different detection methods

One of the simplest and most popular non-radioactive DNA modification is biotinylation which has been used to label cloned DNA probes by nick translation or random priming and to label oligonucleotide probes by 3' tailing with biotin-dUTP or chemical method. We compared the sensitivity of the FISH using biotinylated probe with that of direct fluorophore-labeled probe. The 28S rRNA probe was labeled with biotin N-hydroxy succinimide ester and with Cy5.18. Cy5.18-labeled avidin or streptavidin was used for detecting the hybrids formed with biotinylated probe. Table 2 shows the relative fluorescence of RNase-treated and non-treated cells hybridized with Cy5.18-conjugated or biotin-conjugated 28S rRNA probe. It is clear that directly labeled probe gave the least background and the best signal-to-background ratio. More heavily labeled Cy5-avidin or Cy5-streptavidin gave higher signals, however, the background was also increased significantly, reducing the overall signal-to-background ratio. Detection with FITC labeled-avidin or streptavidin yielded the same result (Table 2). We conclude that direct labeling of oligonucleotide probes is the more sensitive approach for flow cytometric FISH (probe generated by nick translation of histone H4 cDNA clone has not been studied for comparison). Direct labeling also simplifies the hybridization procedure by eliminating the secondary reagent. More importantly, it also provides the potential of multicolor analysis. It is also clear that the Cy5-labeled probe provides better signal-to-background ratio than the FITC-labeled probe. Presumably this advantage is due to the lower autofluorescence obtained with long wavelength (642 nm vs. 488 nm) excitation.

To demonstrate multi-sequence detection with the directly labeled probes (Materials and Methods), we made a Cy3-labeled 18S rRNA probe and a OCy3-labeled 28S rRNA probe. For simultaneous 3 color fluorescence acquisition, the 18S and the 28S probes were excited at 488 nm and Cy5 labeled H4 probes was excited at 647 nm. Because of the abundance of the rRNAs, one oligonucleotide probe for each target sequence was enough to obtain fluorescent signals with signal-to-background ratios of 16 for 18S rRNA and 18 for 28S rRNA. DNA content could be added as an additional parameter by staining the cells with Hoechst 33342 or DAPI after hybridization if one has access to a flow cytometer with UV excitation capability. In a related approach, we are using an imaging fluorescence microscope to simultaneously quantify DNA content (DAPI or Hoechst), histone H4 (Cy5), 18S rRNA (Cy3), and 28S rRNA (OCy3) in HeLa cells on microscope coverslips (Yu, et al., unpublished result).

CONCLUSION

The FISH method described in this report is simple, time-efficient and sensitive. It allows detection of mRNAs as short as 380 bases long in individual cells containing at least 500 copies. The method provides a tool to monitor gene expression or the presence of

viral sequences in heterogeneous cell populations where only some of the cells contain the sequence of interest. Several thousand cells can be analyzed per second to detect rare cells containing the target sequence. The use of panels of oligonucleotides labeled with different color fluorochromes correlates the concentrations of different sequences. If fixation and hybridization methods can be developed to prevent loss of RNA from the cells while at the same time exposing the entire sequence to the oligonucleotide probes, we believe that the method described in this work will permit quantification of RNA concentrations within individual cells. Measurement of fluorescence signals from HeLa cells on microscope slides by image cytometry provides another powerful approach for multicolor analysis of RNA sequences in individual cells (Yu et al., unpublished results). For these reasons, the method reported here should find many applications in biological research and clinical diagnostics.

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REFERENCES

- Lawrence, J.B., Villnave, C.A. and Singer, R.H. (1988) *Cell*, **52**, 51–61.
- Pinkel, D., Straume, T. and Gray, J.W. (1986) *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 2934–2938.
- Lichter, P., Tang, C.C., Call, K., Hermanson, G., Evans, G.A., Housman, D. and Ward, D.C. (1990) *Science*, **247**, 64–69.
- Albertson, D.G., Fishpool, R., Sherrington, P., Nacheva, E. and Milstein, C. (1988) *EMBO J.*, **7**, 2801–2805.
- Viegas-Pequignot, E., Dutrillaux, B., Magdelenat, H. and Coppey-Moisand, M. (1989) *Proc. Natl. Acad. Sci. U. S. A.*, **86**, 582–586.
- Bhatt, B., Burns, J., Flannery, D. and O'D. McGee, J. (1988) *Nucleic Acids Res.*, **16**, 3951–3961.
- Trask, B., Pinkel, D. and van den Engh, G. (1989) *Genomics*, **5**, 710–717.
- Lawrence, J.B., Singer, R.H. and Marselle, L.M. (1989) *Cell*, **57**, 493–502.
- Bauman, J.G.J., Wiegant, J. and van Duijn, P. (1981) *J. Histochem. Cytochem.*, **29**, 238–246.
- Lawrence, J.B. and Singer, R.H. (1986) *Cell*, **45**, 407–415.
- Bauman, J.G.J. and Bentvelzen, P. (1988) *Cytometry*, **9**, 517–524.
- Bayer, J.A. and Bauman, J.G.J. (1990) *Cytometry*, **11**, 132–143.
- Trask, B., van den Engh, G., Landegent, J., Jansen in de Wal, D. and van der Ploeg, M. (1985) *Science*, **230**, 1401–1403.
- Trask, B., van den Engh, G., Pinkel, D., Mullikin, J., Waldman, F., van Dekken, H. and Gray, J. (1988) *Hum. Genet.*, **78**, 251–259.
- Dudin, G., Cremer, T., Schardin, M., Hausmann, M., Bier, F. and Cremer, C. (1987) *Hum. Genet.*, **76**, 290–292.
- Unger, E.R., Hammer, M.L. and Chenggis, M.L. (1991) *J. Histochem. Cytochem.*, **39**, 145–150.
- DeBiasio, R., Bright, G., Ernst, L., Waggoner, A.S. and Taylor, D.L. (1987) *J. Cell Biol.*, **105**, 1613–1622.
- Jensen, R.H. and Leary, J.F. (1990) In *Flow Cytometry and Sorting*. Wiley-Liss, New York, ed. 2, pp. 553–562.
- Heintz, N., Sive, H.L. and Roeder, R.G. (1983) *Mole. Cell. Biol.*, **3**, 539–550.
- Smith, L.M., Fung, S., Hunkapiller, M.W., Hunkapiller, T.J. and Hood, L.E. (1985) *Nucleic Acids Res.*, **13**, 2399–2412.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor University Press, Cold Spring Harbor.
- Singer, R.H., Lawrence, J.B. and Villnave, C. (1986) *BioTechniques*, **4**, 230–250.
- Rickles, R., Marashi, F., Sierra, F., Clark, S., Wells, J., Stein, J. and Stein, G. (1982) *Proc. Natl. Acad. Sci. U. S. A.*, **79**, 749–753.
- Detke, S., Lichter, A., Phillips, I., Stein, J.L. and Stein, G.S. (1979) *Proc. Natl. Acad. Sci. U. S. A.*, **76**, 4995–4999.
- Plumb, M., Stein, J.L. and Stein, G.S. (1983) *Nucleic Acids Res.*, **11**, 2391–2410.
- Robbins, E. and Borun, T.W. (1967) *Proc. Natl. Acad. Sci. U. S. A.*, **57**, 409–416.
- Wu, R.S. and Bonner, W.M. (1981) *Cell*, **27**, 321–330.
- Baumbach, L.L., Stein, G.S. and Stein, J.L. (1987) *Biochemistry*, **26**, 6178–6187.