(54) Title: IN VIVO NUCLEIC ACID HYBRIDIZATION METHOD

(57) Abstract

A method of nucleic acid hybridization in living cells is described, which is useful for detecting, quantitating and locating a specific nucleic acid in a cell or tissue, for selecting cells based on the expression or presence of a specific nucleic acid, and for monitoring the amount and location of a specific nucleic acid over time or under various inducing or inhibiting conditions.
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IN VIVO NUCLEIC ACID HYBRIDIZATION METHOD

Background

In situ hybridization is a useful method for detecting specific nucleic acid sequences in a tissue or individual cell. It has been used to localize specific sequences on chromosomes, to localize viral or cellular mRNAs in cultured cells and tissue sections, and to determine the expression of genes during embryonic and larval (e.g. Drosophila) development (Ausubel et al., eds., Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley-Interscience, New York, 1988, Vol. 2, pp. 14.3.11-14.3.12). In this method, the tissue is fixed and sectioned before applying the hybridization probe. The tissue preparation methods commonly used are cryosectioning, paraformaldehyde fixation and paraffin wax embedding, and plastic sectioning (ibid, pp. 14.1.7-14.1.8, pp. 14.2.6-14.2.8, and pp. 14.3.11-14.3.13). These techniques are intended to preserve cellular morphology while leaving the cellular content and location of nucleic acids intact. However, they kill the cells, with the result that hybridization is actually performed on dead cells. Fixatives penetrate the cells slowly, and it is possible that, during fixation, the nucleic acids rearrange in an artifactual manner. These artifacts may involve such processes as diffusion, degradation, disassembly, polymerization or disassociation. A method allowing hybridization in vivo (i.e. in living cells) would be advantageous.

Influx and efflux of oligonucleotide phosphorothioates in hematopoletic cells for the purpose of future usefulness in antisense research has been
described (Marti et al., Antisense Research and Development 2:27-29, 1992).

Summary of the Invention

This invention relates to a method of nucleic acid hybridization performed in vivo, that is on living cells. According to this method, modified oligonucleotide probes are taken up rapidly and quantitatively by living cells, hybridize to complementary nucleic acids in the cells, and excess probes is washed from the cells, permitting detection and quantitation of specific hybridization within the living cells. The probes are single-stranded oligonucleotides that are modified to increase their ability to penetrate through hydrophobic cellular membranes. The probes are also labelled with a detectable marker, such as a fluorochrome. As described herein, such modified and labelled oligonucleotides appear to enter living cells by passive diffusion.

As further described herein, this hybridization method has no detectable adverse effects on the growth or morphology of living cells. Thus, cells or tissues can be characterized for the presence of a specific RNA or DNA sequence (referred to as the target sequence) without killing the cells. This is presumably because the probes eventually are degraded, diffuse out of the cell, or are diluted by newly synthesized target sequences. This procedure permits the selection of cells or subpopulations of cells based on gene expression or the presence of a target sequence. After hybridization, the cells of interest can be evaluated with respect to other parameters and segregated by methods such as microdissection or flow sorting. The
selected cells can be cultured, further characterized or used as a source of RNA for the construction of cDNA libraries.

In vivo hybridization also permits the expression of a target sequence to be monitored over time and under inducing or inhibiting conditions. Thus, the causal relationships between biochemical or other regulatory factors (e.g. hormones, growth factors, oncogenes, stress conditions) and gene expression can be directly observed in a cell or tissue. The location and accumulation or degradation of a specific nucleic acid sequence can also be monitored. One application of this method is for optimizing antisense oligonucleotide pharmaceuticals. The method can be used to test various antisense oligonucleotide analogs for ability to permeate cellular membranes and hybridize to the target DNA or RNA. Finally, this hybridization method provides a reliable method for locating a target nucleic acid sequence in a living cell or tissue.

Methods for modifying oligonucleotides in order to increase their ability to penetrate cell membranes are known in the art. Such modifications include replacement of phosphate backbone oxygens with, for example, sulfur or methyl groups, to decrease the hydrophilicity of the oligonucleotides and covalent attachment of chemical blocking groups at the 3'-end of the oligonucleotide. Some of these modifications also increase the resistance of the oligonucleotide probes to nucleases. Various detectable markers, labelling methods and methods of detection are also known in the art.
Brief Description of the Drawings

Figure 1 shows the uptake of the probe $^{32}$P-ST30a into living human fibroblast cells.

Figure 2 shows the efflux of the $^{32}$P-ST30a probe from living rat myoblast (L6 cells) cells during the wash step.

Detailed Description of the Invention

Described herein is a method of nucleic acid hybridization that is performed in living cells (in vivo). The cells are not lysed or fixed, as in previous methods of nucleic acid hybridization, and are left apparently unharmed by the hybridization process, as determined by subsequent culture. The method is useful for detecting the presence, amount and location of a specific nucleic acid sequence (referred to as the target sequence) in individual cells or in tissues. The expression of a specific gene can also be assayed. The target nucleic acid sequence can be RNA or DNA. It can be in the nucleus, cytosol, or an organelle of the cell.

The target sequence can be native or foreign to the cell. For example, the presence of sequences specific to virus or bacteria infecting a cell or tissue can be detected. This method is applicable to various cell and tissue preparations, including cultured cells, suspended cells, cell monolayers, live tissue sections, and embryos.

This method employs modified single-stranded oligonucleotides as probes. The oligonucleotides have a sequence that is complementary to the target nucleic acid sequence. The oligonucleotides can be RNAs or DNAs in the approximate size range of 10-50 nucleotides, but
a 15-30 nucleotide length is preferred for specificity and cost-effectiveness. The oligonucleotides are chemically synthesized and modified by known methods to increase their ability to penetrate hydrophobic cellular membranes. Such modifications include substitution of oxygens in the phosphate backbone to decrease the hydrophilicity of the oligonucleotides (Uhlmann and Peyman, Chemical Reviews 90(4):544-584 (1990); Goodchild, Bioconjugate Chemistry 1(3):165-187 (1990)).

For example, one or more of the oxygens in the phosphate backbone can be replaced with sulfur to create phosphorothioate or a phosphorodithioate oligonucleotides (Marshall et al., Proc. Natl. Acad. Sci. USA 89: 6265-6269 (1991)) or with methyl groups to produce methylphosphonate oligonucleotides (Kean et al., Biochem. 27: 9113-9121 (1988)). Other oligonucleotide modifications include sulfone derivatives, formacetal derivatives, peptido-nucleic acids, bicyclo derivatives and methylene acetal derivatives (e.g. Jones et al., NAMA conference, CANCUN, Jan. 1993, p.27; Nielsen et al., Science 254:1697 (1991); Jablonski et al., Nucleic Acids Res. 14:6115 (1986); Smith et al., Nucleic Acids Res. 13:2399 (1985)). In addition, oligonucleotides can be modified by covalent attachment of chemical blocking groups to their 3'-ends. Such 3' blocking groups include amino-modified nucleotides, methyl blocking groups, fluorochrome-conjugated groups, 3' to 5' nucleotide linkages, and succinyl ester groups (e.g. Zerial et al., Nucleic Acids Res. 15:9909-(1987); Li et al, Nucleic Acids Res. 15:5275 (1987); Zuckerman et al., Nucleic Acids Res. 15:5305 (1987)).

Some of these phosphate backbone and 3'-end blocking modifications also render the oligonucleotides
more resistant to cellular nucleases, thus, increasing the effective concentration of the probe in the cell. Further optimization of the probes can be done in order to increase their speed of penetration into and exit from the cell.


Various embodiments of the method are useful for detecting the presence of a target nucleic acid sequence in a living cell or tissue. One embodiment comprises incubating the cell or tissue with a probe under conditions suitable for specific hybridization of the probe to complementary nucleic acid sequences in the cell or tissue, washing the cell or tissue to allow the excess unhybridized probe to exit the cell(s), and
detecting specific hybridization of the probe, which indicates the presence of the target sequence in the cell or tissue. The conditions of hybridization, including concentration of probe, time of hybridization and time of washing, depend on the abundance of target sequence and can be determined empirically through routine experimentation. Preferably, the minimum concentration of probe required to detect specific hybridization in a reasonable amount of time is used. This has been found to be lower by at least three orders of magnitude than the concentrations currently used in the art to obtain inhibition of expression by antisense oligonucleotides. Typically, the higher the concentration of probe, the less incubation time required for hybridization, and vice versa. Too high a concentration of probe, however, increases nonspecific background and is toxic to the cell. Optimization of these conditions can be done by routine experimentation.

The probe is added in a physiological medium acceptable to the cells, such as cell growth medium (preferably without serum). Unlabelled probe with a different sequence can also be added, either with the labelled probe or in the wash medium to reduce nonspecific hybridization. Appropriate positive and negative controls should be performed.

In a preferred embodiment, living cells are incubated in medium containing 10-100 nM fluorochrome-labelled modified DNA probe for about two minutes. The medium is removed, fresh medium is added, and the cells are washed for about 1-2 hours. Less incubation and wash times may be required depending on the abundance of the target sequence and the concentration of the probe. The cells can then be observed by microscopy and the
amount of hybridization quantitated by fluorescence measurements. In cultured chick embryo fibroblasts hybridized under these conditions, target mRNAs in the cytoplasm and nucleus were observed to be hybridized to probe by fluorescence microscopy. Excess probe had exited from the cells after about 1-2 hours incubation in medium. No toxic effects on the cells were observed after three days, as the cells continued to divide.

Rapid equilibration of intracellular with extracellular probe concentrations upon adding the probe has been demonstrated (Figure 1; Example 2). The intracellular concentration of probe reached saturation level in about 15 minutes. This suggests a passive entry mechanism of cell uptake rather than endocytosis, which takes hours. It has also been demonstrated that most of the probe was able to exit the cells during the wash step, apparently also by passive diffusion (Figure 2; Example 4). The intracellular probe concentration reached equilibrium after about 2 hours wash. A small fraction of probe remained in the cells, presumably retained there by hybridization to target sequences, which was visualized microscopically (Example 4). Thus, the in vivo hybridization can be performed in a sufficiently short amount of time so as to be convenient and feasible with living cells.

This method of hybridization can be used to quantitate the amount of a particular target sequence in the cell. As described in Example 4, conditions for hybridization can be used such that virtually all target sequences in the cell are saturated with probe. In this example, hybridization with a phosphorothioate probe blocked any subsequent hybridization with probes complementary to the same target sequence.
In addition, the hybridization method can be performed using two or more differentially labelled probes simultaneously. For example, the expression of two different genes can be monitored in the same cell using probes labelled with different colored fluorochromes.

In a second embodiment of the method, the probe is relatively nuclease-sensitive but remains intact long enough in the cell to hybridize to target sequences, whereupon, in this double-stranded form, it becomes nuclease-resistant, while unhybridized probe is degraded. In this embodiment, it is less necessary to incubate the cells in medium to wash out unhybridized probe; the cells can be quickly rinsed to remove extracellular probe.

In yet another embodiment of the method, energy transfer is used to detect the hybridization. In this case, two modified oligonucleotides, one labelled with an energy donor group and one labelled with an energy acceptor group, comprise the probe. The two oligonucleotides have sequences such that they hybridize to adjacent sites on the target sequence. When the two oligonucleotides are brought near each other due to hybridization to the target sequence, energy transfer takes place between them. When two fluorophores whose excitation and emission spectra overlap are in sufficient close proximity, the excited state energy of the donor molecule is transferred to the neighboring acceptor fluorophore. The result is quenching of donor fluorescence, an enhancement of acceptor fluorescence intensity. The cells are irradiated with light at an excitation wavelength, energy transfer occurs, and the emitted light is detected by increase in fluorescence.
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intensity (see, e.g. Cardullo et al., Proc. Natl. Acad. Sci. USA 85:8790-8794 (1988)).

Utility of the Invention

A major advantage of this hybridization method is the ability to detect nucleic acid sequences or evaluate gene expression in a cell or tissue without killing it. The cells of interest are still available after hybridization for further observation and characterization. Thus, cells can be selected based on the expression of any known gene sequence, i.e. presence of the target sequence among the cellular RNAs. The genes can be chromosomal, mitochondrial or chloroplastic, episomal, viral or transfected. For many applications, quantitation of the detected cells is sufficient. However, hybridization can also be followed by physical segregation of the cells of interest by known methods, such as fluorescence activated cell sorting or microdissection. The isolated subpopulation can then be cultured as a subclone or used as a source of RNA for cDNA library construction.

A second advantage of this method is that it adds a temporal dimension to the diagnostic capabilities of nucleic acid hybridization. The expression of a specific gene can be monitored over the course of a cell's or tissue's differentiation or development. The hybridization method can be performed repeatedly on a population of cells without damaging the cells. The effect of regulatory factors, such as other genes, hormones, differentiation, growth protein factors, and environmental conditions, on the expression of specific genes in a cell or tissue can be observed directly. For example, the induction of the transformed phenotype by
expression of an oncogene can be directly observed. This method obviates the often difficult task of obtaining homogeneous and synchronized cell populations for studies on gene expression. Since hybridization of the DNA probe may result in destruction of the endogenous RNA by endogenous RNAse H, which digests the RNA in DNA-RNA duplexes, the cellular response to destruction of this RNA could be monitored directly.

The in vivo method is expected to be more informative than previous hybridization methods for locating target nucleic acids in cells or tissues, since live cells are used in the assay. In addition, the method is quicker and simpler than hybridization methods requiring pretreatment of the cells with fixatives, and subsequent in situ hybridization, which can take hours.

This method is also useful for determining the presence of specific DNA sequences in cells. In this case, the single-stranded probe forms a triple helix (C=G=O or T=A=T) with the target double-stranded DNA.

For example, retroviral sequences, such as from the Human Immunodeficiency Virus (HIV), can be visualized and their presence in the chromosome (e.g. at the site of transcription) or in nuclear or cytoplasmic RNA can be monitored. Gross chromosomal deletions, additions and translocations may also be identified by triple helix formation, for example, for diagnosis of trisomy 21 in Down's Syndrome or deletions in the X-chromosome in muscular dystrophy, where the loss of hybridization signal to dystrophin would indicate the loss of the mRNA or the gene. The presence and movements of infectious agents (viral, bacterial, mycoplasma) and parasites, or their nucleic acids, can also be observed in tissues and organs. Movements of chromatin can be monitored during
changes in cell behavior (e.g. transformation) or during development.

In addition, the in vivo hybridization method is useful for assessing and optimizing pharmaceuticals based on antisense oligonucleotides. Antisense nucleotides are currently being developed, for instance, as an anti-HIV therapy. It is known in the art that some antisense probes or procedures are unsuccessful or inefficient. The ability to visualize these molecules in vivo would aid in the analysis of parameters and molecule design that are important for successful inhibition of HIV. For example, the ability of various antisense probes to enter the nucleus and hybridize to sequences in the cell can be empirically assessed.

The following examples more specifically illustrate the invention.

Example 1

**Synthesis of Fluorochrome-labelled Phosphorothioate Probes**

Oligonucleotide (30 nucleotides) probes containing phosphorothioate linkage in each position were made on the Applied Biosystem DNA Synthesizer 396 (Foster City, CA) with amino-modified linker thymidines added at the second positions from the 3′- and 5′-ends. After purification by chromatography on Sephadex G50, the DNA was labelled with fluorochromes (Texas red, Cy3 or FITC) in NaHCO₃/Na₂CO₃, pH 9.0, overnight at room temperature in the dark (Molecular Probes, Eugene, OH). Labelled oligonucleotides were purified by electrophoresis on 10%
polyacrylamide gel under non-denaturing conditions and eluted from the appropriate bands.

Example 2
Quantitative Analysis of Probe Uptake Into Cells

The kinetics of uptake of modified single-stranded oligonucleotide probes into living cells was examined using a phosphorothioate poly(dT) oligonucleotide of thirty nucleotides length, referred to as ST30a. This oligonucleotide was synthesized, as described above, with an amino linker for fluorochrome attachment. For the purposes of this experiment, however, the oligonucleotide was labelled with $^{32}$P. The poly(dT) is expected to hybridize intracellularly to the poly(A) tracts of the mRNAs in the cell. Approximately 20,000 human fibroblast cells were exposed to an extracellular concentration of 0.1 $\mu$M of the $^{32}$P-labelled probe for 2 hours and the intracellular concentration of the probe determined after 0, 2, 5, 10, 30, 60 and 120 minutes incubation (Figure 1). The intracellular probe concentration was determined by measuring the Cerenkov radiation of the cells (after rinsing them) in a scintillation counter.

As shown in Figure 1, cells took up the $^{32}$P-ST30a probe very rapidly in the first about 5 minutes, reaching saturation in about 15 minutes. The peak intracellular concentration of probe was about 280 pg per 20,000 cells or 0.1 $\mu$M. The rapidity with which the internal probe concentration equilibrated with the extracellular probe concentration (less than 10 minutes) suggests that the oligonucleotide probes go into the
cell passively, rather than by endocytosis, which takes hours, as previously reported.

The intracellular concentration of probe can be calculated as follows:

280 pg probe/20,000 cells = approx. $10^{-2}$ pg/cell.

Given that 1 cell = approx. 10 pl,

$10^{-2}$ pg/cell = $10^{-2}$ pg/10 pl = $10^{-3}$ pg/pl = 0.1 μM.

The volume per cell is calculated as follows:

Diameter of one cell = approx. 20 microns.

Volume of one cell = approx. $(20 \, \mu m)^{3} = 10^{4} \, \mu m^{3} = 10$ pl.

**Example 3**

Exit of the Probe From Cells

The ability of the probe to exit cells was examined by incubating about $5 \times 10^{4}$ rat L6 myoblasts in about 0.1 μM $^{32}$P-ST30a for about 10 minutes, removing the probe, and washing the cells in fresh medium without probe. Figure 2 shows the amount (pg) of intracellular probe during washing of the cells. After about 2 hours, the intracellular probe dropped to an equilibration level of about 100 pg or $5 \times 10^{4}$ pg/cell. This experiment shows that a majority of the probes entering the cells exited the cells during the wash. It is reasonable to conclude that the nonequilibrating fraction of probe (about 100 pg) is retained in the cells by hybridization to target sequences.

In order to saturate target sequences in the cells, the hybridization experiment was also performed with 15 μM of unlabelled ST30a containing a tracer amount (0.1
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\( \mu \text{M} \) of the \(^{32}\text{P}\)-labelled probe. As before, cells were incubated with probe for 10 minutes, the probe removed, and the cells washed with fresh medium for 2 hours. A separate experiment comprising a 30 minute incubation with probe and 2 hour wash was also performed with similar results. After the wash, both experiments yielded a final intracellular concentration of about 38 pg probe within the cells. These numbers correlate well with the expected amount of poly(A) target sequences within the cell, about 100,000 molecules. Since the oligonucleotide is 30 nt long, 30 x 330 (mol. weight of one mole of nucleotide) = 10^4 grams/mole of oligonucleotide. Therefore, about 40 picograms in 20,000 cells = 40 x 10^{-12} g, or 20 x 10^{-16} g in one cell.

Since 10^4 grams = 6 x 10^{23}, one oligonucleotide = 0.16 x 10^{-19} g. Therefore, 20 x 10^{-16} g/0.2 x 10^{-19} g = 100,000 molecules hybridized per cell. This is very close to a complete hybridization of intracellular targets.

Example 4

Microscopic Analysis of In vivo Hybridization

A direct observation of hybridization of the probes within cells was made by fluorescent microscopy. Cells were exposed to a red fluorochrome-conjugated phosphorothioate poly(dT) (30 nucleotides long) probe. Hybridization was carried out, with 10 minutes incubation in 1 \( \mu \text{M} \) probe and 2 hours wash. After the wash, the cells were fixed and examined by fluorescent microscopy. Red fluorescence was observed in the nucleus and diffusely throughout the cytosol of the cells, indicating hybridization of the probes to RNAs in

The fixed cells were further hybridized to a green poly(dT) probe (not a phosphorothioate). Hybridization of the fixed cells was carried out with 1 μM green probe in 2X SSC, 15% formamide for 1 hour and washed three times for 5 minutes each. No hybridization of the poly(dT) probe was detected, indicating that the target sequences were not available for hybridization, i.e. they were already hybridized to the red probe.

In a control experiment, in vivo hybridization to a phosphorothioate poly(dC) (30mer) did not block subsequent hybridization with the poly(dT) green probe, indicating that the hybridization observed with the phosphorothioate poly(dT) probe was sequence specific. These results demonstrate that sequence specific hybridization can be performed in vivo.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.
The invention claimed is:

1. A method for detecting target nucleic acids in a living cell or tissue, such method being performed without adverse effects on the growth or morphology of the living cell or tissue, comprising the steps of:
   a) incubating a living cell or tissue with a labelled, modified single-stranded oligonucleotide probe under conditions suitable for specific hybridization of the probe to complementary nucleic acids in the cell or tissue, said oligonucleotide having a nucleotide sequence complementary to the target nucleic acids and modified to increase its ability to penetrate cellular membranes;
   b) washing the cell or tissue to allow the unhybridized probe to exit from the cell or tissue; and
   c) detecting specific hybridization of the probe, wherein specific hybridization indicates the presence of the target nucleic acids in the cell or tissue, thereby detecting target nucleic acids in the living cell or tissue without adverse effects on the growth or morphology of the living cell or tissue.

2. The method of Claim 1, wherein the modified oligonucleotide has at least one substitution of a phosphate backbone oxygen that decreases the
hydrophilicity of the oligonucleotide compared to an unmodified oligonucleotide.

3. The method of Claim 2, wherein the modification of the oligonucleotide is selected from the group consisting of:
   a) phosphorothioate;
   b) phosphorodithioate;
   c) methylphosphonate;
   d) formacetal derivative;
   e) sulfone derivative;
   f) peptido-oligonucleotide;
   g) bicyclo derivative; and
   h) methylene acetal derivative.

4. The method of Claim 1, wherein the probe is an oligonucleotide with a 3'-end blocking group.

5. The method of Claim 4, wherein the 3'-end blocking group is selected from the group consisting of:
   a) amino-modified nucleotide;
   b) methyl group;
   c) fluorochrome-conjugated group;
   d) 3' to 5' nucleotide linkage; and
   e) succinyl ester group.

6. The method of Claim 1, wherein the probe is labelled with a fluorochrome.

7. The method of Claim 1, wherein in step a), two or more differentially labelled probes are used, and wherein each said probe has a nucleotide sequence complementary to different target nucleic acids.
8. The method of Claim 1, wherein in step a), the cell or tissue is incubated with the labelled probe and an unlabelled oligonucleotide with a nucleotide sequence different from the nucleotide sequence of the labelled probe, wherein the unlabelled oligonucleotide has the same modification as the labelled probe.

9. The method of Claim 1, wherein in step b), the cell or tissue is washed in medium containing unlabelled probe which has a nucleotide sequence that is not complementary to the target nucleic acids.

10. The method of Claim 1, wherein the target nucleic acid are RNAs.

11. The method of Claim 1, wherein the target nucleic acids are from an infectious agent.

12. The method of Claim 11, wherein the infectious agent is a virus.

13. The method of Claim 1, wherein the target nucleic acids are antisense oligonucleotides exogenous to the cell.

14. The method of Claim 1, wherein detecting specific hybridization of the probe comprises a method selected from the group consisting of:
   a) fluorescence microscopy;
   b) flow cytometry; and
   c) image analysis.
15. The method of Claim 1, wherein detecting specific hybridization of the probe comprises quantitating the amount of probe specifically hybridized, wherein the amount of probe specifically hybridized indicates the amount of the target nucleic acids in the cell or tissue.

16. A method for detecting target nucleic acids in a living cell or tissue, such method being performed without adverse effects on the growth or morphology of the living cell or tissue, comprising the steps of:
   a) incubating the cell or tissue in a physiologically acceptable medium containing 10-100 nM of a fluorochrome-labelled, single-stranded phosphorothioate oligonucleotide for about 2-30 minutes;
   b) removing the medium containing the probe and washing the cell or tissue in medium without probe for about 1-2 hours; and
   c) detecting fluorescence of the probe in the cell or tissue, wherein fluorescence indicates the presence of the target nucleic acids in the cell or tissue, thereby detecting target nucleic acids in a living cell or tissue without adverse effects on the growth or morphology of the cell or tissue.

17. A method for detecting target nucleic acids in a living cell or tissue, such method being performed without adverse effects on the growth or morphology of the living cell or tissue, comprising the steps of:
incubating a living cell or tissue with a labelled, modified single-stranded oligonucleotide probe under conditions suitable for specific hybridization of the probe to complementary nucleic acids in the cell or tissue, said oligonucleotide having a nucleotide sequence complementary to the target nucleic acids and modified to increase its ability to penetrate cellular membranes, said probe which is hydrolyzed by nucleases when not hybridized and is not hydrolyzed by nucleases when hybridized to complementary nucleic acids; and

b) detecting specific hybridization of the probe, wherein specific hybridization indicates the presence of the target nucleic acids in the cell or tissue, thereby detecting target nucleic acids in a living cell or tissue without adverse effects on the growth or morphology of the cell or tissue.

A method for detecting target nucleic acids in a living cell or tissue, such method being performed without adverse effects on the growth or morphology of the living cell or tissue, comprising the steps of:

a) incubating a living cell or tissue with a probe comprising two modified single-stranded oligonucleotides under conditions suitable for specific hybridization of the oligonucleotides to complementary nucleic acids in the cell or tissue, said oligonucleotides which are complementary to immediately adjacent sites on
the target nucleic acid sequence and modified to increase their ability to penetrate cellular membranes, the oligonucleotide with its 5' end closest to the 3' end of the second oligonucleotide when hybridized to immediately adjacent sites on the target nucleic acid sequence being labelled on the 5' end with either an energy donor group or an energy acceptor group and the second oligonucleotide being labelled on the 3' end with whichever group, either an energy donor group or an energy acceptor group, not being used as a label on the first oligonucleotide;
b) washing the cell or tissue to allow the unhybridized probe to exit from the cell or tissue; and
c) detecting energy transfer between the two oligonucleotides, wherein energy transfer indicates the presence of the target nucleic acids in the cell or tissue, thereby detecting target nucleic acids in a living cell or tissue without adverse effects on the growth or morphology of the cell or tissue.

19. A method for detecting expression of target nucleic acids in a living cell or tissue, such method being performed without adverse effects on the growth or morphology of the living cell or tissue, comprising the steps of:
a) incubating a living cell or tissue with a labelled, modified single-stranded oligonucleotide probe under conditions suitable for specific hybridization of the
probe to complementary RNAs nucleotides in the cell or tissue, said oligonucleotide having a nucleotide sequence complementary to the target nucleic acids and modified to increase its ability to penetrate cellular membranes;

b) washing the cell or tissue to allow the unhybridized probe to exit from the cell or tissue; and

c) detecting specific hybridization of the probe to RNA in a cell or tissue, wherein specific hybridization indicates that the cell or tissue expresses the target nucleic acids, thereby detecting expression of target nucleic acids in a living cell or tissue without adverse effects on the growth or morphology of the cell or tissue.

20. The method of Claim 19, wherein detecting specific hybridization of the probe comprises quantitating the amount of probe specifically hybridized, wherein the amount of probe specifically hybridized indicates the level of expression of the target nucleic acids in the cell or tissue.

21. A method for monitoring the expression of target nucleic acids in a living cell or tissue, such method being performed without adverse effects on the growth or morphology of the living cell or tissue, comprising performing the method of Claim 19 more than once.

22. A method for selecting a cell that expresses target nucleic acids, such method being performed without
adverse effects on the growth or morphology of the cell, comprising the steps of:

a) incubating a population of cells with a labelled, modified single-stranded oligonucleotide probe under conditions suitable for specific hybridization of the probe to complementary RNAs nucleotides in the cells, said oligonucleotide having a nucleotide sequence which is complementary to the target nucleic acids and modified to increase its ability to penetrate cellular membranes;

b) washing the cells to allow the unhybridized probe to exit from the cell; and

c) detecting specific hybridization of the probe to RNAs in a cell, wherein specific hybridization indicates that the cell expresses the target nucleic acids, thereby selecting a cell that expresses target nucleic acids without adverse effects on the growth or morphology of the cell.

23. The method of Claim 22, further comprising separating the expressing cell from nonexpressing cells in the cell population.

24. The method of Claim 23, wherein the separating step comprises a method selected from the group consisting of:

a) flow sorting; and

b) microdissection followed by subcloning.
25. A method for locating target nucleic acids in a living cell or tissue, such method being performed without adverse effects on the growth or morphology of the cell or tissue, comprising the steps of:

a) incubating a living cell or tissue with a labelled, modified single-stranded oligonucleotide probe under conditions suitable for specific hybridization of the probe to complementary nucleic acids in the cell or tissue, said oligonucleotide having a nucleotide sequence which is complementary to the target nucleic acids and modified to increase its ability to penetrate cellular membranes;

b) washing the cell or tissue to allow the unhybridized probe to exit from the cell or tissue; and

c) detecting specific hybridization of the probe, wherein detection of the specific hybridization indicates the location of the target nucleic acids in the cell or tissue, thereby locating target nucleic acids in a living cell or tissue without adverse effects on the growth or morphology of the cell or tissue.
FIG. 1
### A. CLASSIFICATION OF SUBJECT MATTER

**IPC 6** C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

**IPC 6** C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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**X** Further documents are listed in the continuation of box C.  

**X** Patent family members are listed in annex.

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**Date of the actual completion of the international search**

27 October 1994

**Date of mailing of the international search report**

- 9. 11. 94

**Name and mailing address of the ISA**

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**Authorized officer**

Hornig, H
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### Information on patent family members

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