Fluorescence In Situ Hybridization

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Overview

Fluorescence In Situ Hybridization

probe DNA

Labeling with fluorescent dye

Denature & Hybridize

X chromosome
Probe Selection

*The probe must be selected with extreme care.

Oligonucleotide: typically 15-30 bp
  Pro: easy access to target
  Con: carry fewer labels--less fluorescence

Polynucleotide: -100 bp
  Pro: high fluorescence
  Con: non-specific binding
Labeling: Direct

A. Amino linker 5’
B. Terminal transferase at 3’ *(can use both A&B)

Benefits:
Commercially available
Can be stored for several months
Fast, cheap and easy
No additional steps

BUT: Low Sensitivity

Labeling: Indirect

C. Linked Reporter molecule (DIG), which is detected by a fluorescent antibody (8X brighter than direct)

D. Horseradish peroxidase- made 10-20X brighter, but: less cells were positive because of large molecules. Lysozyme-but not for mixed pops. (TSA or CARD)

E. Polynucleotide w/ DIG

Simultaneous observation of 2 colors can be done by using multibandpass filters (MC-FISH).

Select fluorochromes with sharp emission peaks to avoid overlap and background.

Dapi can be used for counterstaining.

**Table:**

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Wavelength</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMCA</td>
<td>351</td>
<td>Blue</td>
</tr>
<tr>
<td>Fluorescein–isothiocyanate (FTTC)</td>
<td>492</td>
<td>Green</td>
</tr>
<tr>
<td>5-(-6-)carboxyfluorescein–N-hydroxysuccinimide-ester (FluoX™)</td>
<td>488</td>
<td>Green</td>
</tr>
<tr>
<td>Tetramethyl–rhodamine–isothiocyanate (TRITC)</td>
<td>557</td>
<td>Red</td>
</tr>
<tr>
<td>Texas Red™</td>
<td>578</td>
<td>Red</td>
</tr>
<tr>
<td>Cyanine Dyes</td>
<td>550</td>
<td>Orange/red</td>
</tr>
<tr>
<td>Cy3™</td>
<td>570</td>
<td>Orange/red</td>
</tr>
<tr>
<td>Cy5™</td>
<td>674</td>
<td>Infrared</td>
</tr>
</tbody>
</table>
Target: Why Use rRNA?

- Genetic stability
- Structure (conserved and variable regions)
- High copy number
- Probes can be designed to from kingdom to species taxon level
- Large data base of sequences available
**Fixation:** Usually alcohol or formaldehyde. Optimize for probe penetration, retention of RNA and structural integrity.

**Sample:** may be treated for G+. Treat glass slide (gelatin)

**Hybridization:** Stringency determined by probe sequence. Adjust by Temp or Formamide.

**Washing:** remove unbound probes

**Visualization:** many microscopes

Pitfalls: False Positives

**Autofluorescence**
- molds, yeast, certain bacteria-
  check before FISH

**Lack of probe specificity**
- use + control, and closely related species for - control
Pitfalls: False Negatives

- Secondary or Tertiary Structure
  - Check for hairpin loops and self-annealing
- Insufficient probe penetration
- Low rRNA concentration
- Photobleaching
  *** Use a Eubacterial Probe
FISH modifications

- **PNA (peptide nucleic acids)** -
  - Uses a backbone made of polyalamide for probes, resulting in more stable hybrids.

- **Micro-CARD-FISH** -
  - A more sensitive FISH technique that incorporates Microradioautography - a complex method that measures the uptake of labeled nutrients such as glucose, amino acids or acetate.
FISH and Flow Cytometry

- Flow cytometers are designed to pass individual cells through focused laser beams.
- The optical properties of each cell are measured and recorded as they pass through the laser.
- The flow cytometer allows rapid counts and identification of different cell types in culture and field samples, based upon fluorescence of applied molecular probes.
- Some can sort cells
FISH Applications

- FISH is VERY widely used
  - Medicine
  - Endosymbionts
  - Mixed Communities
  - Metabolic properties
  - Waste water
  - Viral and Bacterial infections
  - And many more.....
Protozoan with bacterial endosymbionts.

Blue: Dapi

Red: probe for α-proteobacteria,

Pink: co-localization

Green: autofluorescence
Fluorescence in situ hybridization of subgingival plaque from a periodontitis patient.

(a) Simultaneous hybridization with eubacterial probe EUB338FITC (green) for visualization of different bacterial morphologies at single-cell resolution and TRE ICy3 (yellow) for the detection of phylogenetic group I treponemes, most of which are as-yet uncultured.

(b) The same plaque material, hybridized with TRE IFITC (green) and TRE IIICy3 (yellow), the latter detecting oral treponemes of phylogenetic group II
FIG. 1. Effect of the addition of competitor nonfluorescent-oligonucleotide probes on the specificity of hybridization. Histograms show the distribution of green fluorescence (in arbitrary units [a.u.]) of *P. canavese* (A and C) and *C. coccineus* (B and D) cells after hybridization with 2.5 ng of FITC-CHLO or FITC-NCHLO µL⁻¹ in the absence (A and B) and in the presence (C and D) of 2.5 ng of competitor unlabeled probes µL⁻¹. Shaded histograms show the autofluorescence of fixed cells incubated in hybridization buffer without probe (control).
FIG. 2. Variation of cell fluorescence normalized to the fluorescence of 0.95-μm-diameter beads during the growth of the nanoplanktonic species *P. carnea* (A and C) and *C. coccola* (B and D). (A and B) \( F_{sp}/F_{nsp} \). (C and D) Corresponding variations of cell autofluorescence (ctrl), \( F_{sp} \) (NCHLO for *P. carnea* and CHLO for *C. coccola*), and \( F_{nsp} \) (CHLO for *P. carnea* and NCHLO for *C. coccola*). a.u., arbitrary units.
FIG. 3. Fluorescence signals measured after hybridization of *P. carinii* cells in exponential (A) or stationary (B) phase. Histograms show the distribution of green fluorescence for cells hybridized with the CHLO and NCHLO probes and the autofluorescence of control cells. Note that specifically hybridized cells were completely separated from nonspecifically hybridized cells during exponential phase. In stationary phase, the overlap between the CHLO and NCHLO histograms involved only 1% of the total cell number. a.u., arbitrary units.
FIG. 4. Variation of cell fluorescence normalized to the fluorescence of 0.85-μm-diameter beads during the growth of the picoplanktonic species *P. calcocatala* (A and C) and *Chlorella* sp. (B and D). (A and B) $F_{sp}/F_{esp}$, (C and D) Corresponding variations of cell autofluorescence (ctrl). $F_{esp}$ (NCHLO for *P. calcocatala* and CHLO for the *Chlorella* sp.), and $F_{esp}$ (CHLO for *P. calcocatala* and NCHLO for the *Chlorella* sp.). a.u., arbitrary units.
FIG. 6. Fluorescence signals measured after hybridization of *C. trachomatis* cells in exponential (A) or stationary (B) phase. Histograms show the distribution of green fluorescence for cells hybridized with the CHLO and NCHLO probes and the autofluorescence of control cells. The overlap between the histograms involved only 4% of the cells for exponentially growing organisms (A), compared with 54% of the cells in stationary phase (B). a.u., arbitrary units.
FIG. 1. Percentages of DAPI-stained cells in the German Bight of the North Sea (surface samples, 1998) detected by oligonucleotide (EUB338) and polynucleotide (EUBAC) probes targeted to bacteria. FISH data with the probe EUB338 are from reference 12.
FIG. 2. Percentages of DAPI-stained cells in the upper 200 m of Monterey Bay detected with oligonucleotide and polynucleotide probes for bacteria and archaea, from 12 April 2000 (A) and 16 November 2000 (B). Oligonucleotide probes: ARCH915 (Archaea), EUB338 (bacteria). Polynucleotide probes: G1 (crenarchaeota), G2 (euryarchaeota), EUBAC (bacteria). Probes detecting <1% of DAPI-stained cells throughout the profiles are omitted from the graphs.

FIG. 3. Bleaching dynamics of North Sea picoplankton enrichment stained with the monolabeled oligonucleotide probe EUB338 or the unlabeled polynucleotide probe EURBAC and labeled with fluorescein (A) or Cy3 (B). A signal-to-background ratio of 1 indicates object brightness equivalent to the background fluorescence.
Discussion: Pernthaler et al.

**FIG. 4.** Signal-to-background ratio spectra of cells hybridized with oligonucleotide probe EUB338 (labeled with Cy5) and polynucleotide probe EURAC (labeled with fluorescein). Samples were from Monterey Bay from 12 April 2000 (A) or 16 November 2000 (B). A signal-to-background ratio of 1 indicates object brightness equivalent to the background. For (A), Cy3-labeled probe EUB338 was used.
FIG. 1. Dependence of the percentage of cells taking up 1-Asp (cells with more than three associated silver grains related to the abundance of DAPI-stained cells) on the duration of the exposure for autoradiographic development. Prokaryotic assemblages collected from a 100-, 2,700-, or 2,750-m depth were analyzed.

FIG. 2. Comparison of the detection rates (percentage of probe-hybridized cells normalized to total DAPI-stained cells) by CARD-FISH for Crenarchaeota (Cren357) and Euryarchaeota (Eury806) when using lysozyme and proteinase K treatment for cell wall permeabilization.
FIG. 3. Percentages of Bacteria, Crenarchaeota, and Euryarchaeota normalized to total DAPI counts (A) and percentages of Bacteria, Crenarchaeota, and Euryarchaeota taking up L-Asp normalized to the total number of Bacteria, Euryarchaeota, and Crenarchaeota (B), respectively, in different depth layers of the North Atlantic. Bars represent the mean (= standard error) of data from 10 different stations.