Catalyzed Reporter Deposition - Fluorescence In Situ Hybridization (CARD-FISH)
Rappé Lab Protocol

(version 28May2013 prepared by M. Rappé, with text borrowed from several other sources)

Fluorescence *in situ* hybridization (FISH) with rRNA-targeted probes is a staining technique that allows phylogenetic identification of bacteria in mixed assemblages by means of epifluorescence microscopy, without prior cultivation.

In theory, each ribosome within a bacterial cell, containing one copy each of 5S, 16S and 23S rRNA, is stained by one probe molecule during the hybridization procedure, the high numbers of ribosomes per cell thus providing a natural signal amplification system. However, in environmental samples, hybridization with oligonucleotides carrying only a single fluorochrome may not provide enough fluorescence signal to detect cells with low ribosome content. An alternative labeling technique that increases fluorescence signals intensity uses horseradish-peroxidase (HRP-) labeled oligonucleotides. When using HRP labeled probes, fluorescent staining results from a secondary incubation with fluorescently labeled tyramide. The specifically bound peroxidase molecules catalyze the deposition of labeled reporter compounds within cells targeted by the HRP tagged probe. Fluorescence intensity can be up to 20-fold higher with HRP labeled probes than with conventional single labeled probes. However, cell permeabilization protocols need to be adjusted in order to enable the larger enzyme-labeled oligonucleotides to penetrate into cells.

Generally, there are three additional steps in CARD-FISH protocols: embedding in agarose, permeabilization, and tyramide incubation. The first is necessary to prevent cell loss due to the added permeabilisation. The second is necessary for allowing the HRP moety to enter the cell, and the third is for the signal amplification. Other differences to “normal FISH” include the addition of dextran sulfate to the hybridization buffer in order to enhance the binding of probe to its target, and the use of “blocking reagent” to decrease nonspecific binding of the HRP. Some microorganisms carry peroxidases or proteins with pseudoperoxidase activity. These enzymes have to be inactivated by treatment with dilute hydrochloric acid or hydrogen peroxide.

Specificity of probe binding to the target site depends on the hybridization and washing conditions. During hybridization, samples are incubated at elevated temperature in an airtight vessel saturated with hybridization buffer to avoid concentrating effects due to evaporation. The washing step serves to remove excess probe molecules under stringent conditions that prevent unspecific binding.

PROTOCOL

1. Sample Fixation (for planktonic bacteria and archaea)

1.1. In an amber polypropylene bottle, fix samples with fresh 20% electron microscopy-grade paraformaldehyde at a final concentration of 2% for 4-12 hr at 4°C. For microbial cultures, samples can be fixed for 15 min at room temp.

1.2. Filter the samples gently (ca. 5-20 mm Hg) onto 0.1 µm pore-sized, 25 mm diameter white polycarbonate filters. Black membranes work as well, but color may come off during CARD-FISH. Use a Pall GN-4 mixed cellulose membrane filter as a backing membrane for even distribution of cells.

1.3. Wash twice with 3-5 mL of 1X PBS.

1.4. Air-dry the filters on a piece of Whatman paper, label with a lead pencil, and store at -20°C dessicated. Filters can be stored for months (probably years).

2. Embedding Cells in Agarose

2.1. Cut filters into 6 sections (4-8 sections also works) and label each with a pencil. This is tricky. Can also proceed with embedding without cutting the membranes.

2.2. Melt 0.1% (wt/vol) low melting point agarose (0.2 g in 200 mL MilliQ water) in a microwave, let cool in a waterbath set at 40°C.

2.3. Rapidly (but completely) dip both sides of the filter sections into the melted agarose.

2.4. Place the filter sections onto parafilm stretched over a 75x50 mm glass microscope slide, ensuring that the cell side is facing up.

2.5. Let the filters dry in a hybridization oven for 10-30 min at ca. 35°C.

2.6. Pipet ethanol [80-96% (v/v)] onto the filters and carefully peel them off the parafilm.

2.7. Incubate the filters in ethanol [80-96% (v/v)] for 1 min at room temperature.

2.8. Air dry the filters cell-side up on Whatman paper or tissue. The filters can subsequently be stored at -20°C.

3. Permeabilize With Lysozyme (Bacteria)

3.1. Prepare 10-20 mL of a fresh lysozyme permeabilization solution (Appendix 1).

3.2. Pour 1-2 mL of lysozyme solution into a microtiter plate and incubate the filter sections upside down in the solution for 1 hr at 37°C.

3.3. Wash the filters in a petri dish or small beaker filled with MilliQ water 1x.

3.4. Briefly incubate the filters in 0.01M HCl at room temperature, and then wash the filters with MilliQ water 2x.

3.5. Briefly incubate the filters in a petri dish or small beaker filled with 95% ethanol.

3.6. Let filters dry. After permeabilization, the filters can be stored at -20°C for several weeks.

4. Permeabilize With Proteinase K (Archaea)

4.1. Prepare 10-20 mL of a fresh proteinase K permeabilization solution (Appendix 1).

4.2. Pour the proteinase K solution into a small petri dish and incubate the filter sections upside down in the solution for 1 hr at 37°C.

4.3. Wash the filters in a petri dish or small beaker filled with MilliQ water 3x.

4.4. Briefly incubate the filters in 0.01M HCl at room temperature, and then wash the filters with MilliQ water 2x.

4.5. Briefly incubate the filters in a petri dish or small beaker filled with 95% ethanol.

4.6. Let filters dry. After permeabilization, the filters can be stored at -20°C for several weeks.

5. Permeabilize With Achromopeptidase (Gram Positive bacteria)

5.1. Permeabilize with lysozyme first (steps 3.1-3.3).

5.2. Prepare 2 mL of a fresh Achromopeptidase solution (Appendix 1).

5.3. Incubate for 15-30 min at 37°C.

5.4. Wash the filters in a small petri dish filled with MilliQ water 1x.

5.5. Briefly incubate the filters in 0.01M HCl at room temperature, and then wash the filters with MilliQ water 2x.

5.6. Briefly incubate the filters in a petri dish or small beaker filled with 95% ethanol.

5.7. Let filters dry. After permeabilization, the filters can be stored at -20°C for several weeks.

6. Hybridization

6.1. Mix 900 µL of the appropriate hybridization buffer (Appendix 1) with 4.5 µL HRP-labeled 50 ng/µL oligonucleotide probe working stock (0.25 ng/µL final concentraton; Appendix 2). This concentration is standard, but can optimize depending on high background or low probe signal (e.g. 4.5 µL or 0.25 ng/µL).

6.2. Pre-heat the hybridization buffer/probe solution to 35°C, and then place the filters into 450 µL buffer in and eppendorf tube (one filter section per tube).

6.3. Cover the tubes individually with parafilm (to prevent leaking) and then aluminum foil (to block light).

6.4. In a pre-heated hybridization oven, hybridize by shoving in a hybridization tube with paper towels to hold them in place (10 rpm) overnight at 35°C (min. of 4 hr).

6.5. Probes can be frozen once; otherwise store at 4°C for up to 6 months.

7. Wash

7.1. Preheat the appropriate wash buffer to 37°C (probe-specific).

7.2. Remove the filters from the hybridization reaction, lightly touch them to tissue paper to whick away excess hyb buffer, and place them in 5 mL of preheated wash buffer for 10-15 min.

7.3. Remove the filters from the wash buffer by pouring it into a petri dish.

7.4. To equilibrate the probe-delivered HRP, incubate filters in 5 mL of 1X PBS (pH 7.6) for 15 min at room temperature.

7.5. Remove the filters and use immediately in the catalyzed reporter deposition.

7.6. Do not let the filter sections dry out - this reduces the activity of the HRP.

8. Catalyzed Reporter Deposition (using TSA kit from Perkin Elmer; NEL744)

8.1. Prepare the amplification reagent: add Cy3 tyramide to amplification reagent, 1:50. For example, 4 µL Cy3 tyramide plus 200 µL amplification reagent is enough for 6 filter pieces. Amplification reagent is supplied with TSA kit.

8.2. Place 30 µL Cy3 solution per filter piece on parafilm; place filter piece cell side down into the Cy3 solution.

8.3. Incubate at room temperature in the dark for 3-10 min.

8.4. Remove excess liquid by dabbing filters onto blotting paper. Wash sections in a petri dish containing 1X PBS for 15 min at room temperature in the dark.

8.5. Wash sections in MilliQ H_2O for 1 min at room temperature in the dark.

8.6. Wash sections in 95% or absolute ethanol for 1 min at room temperature in the dark.

8.7. Let sections air dry.

8.8. Mount the filter sections on microscope slides with a Citiflour/Vectashield/DAPI mix (Appendix 1).

8.9. If sealed with nail polish, slides may be stored at -20°C until further processing.

APPENDIX 1. SOLUTIONS AND BUFFERS

Lysozyme permeabilization solution (make fresh daily)

Stock	Final conc.	Vol. for 10 mL
0.5 M EDTA pH 8.0	50 mM	1 mL
1 M Tris HCl, pH 8.0	100 mM	1 mL
MilliQ H_2O	--	7 mL
100 mg/mL lysozyme stock (in MilliQ; stored at -20°C)	10 mg/mL	1 mL

Proteinase K permeabilization solution (make fresh daily)

Stock	Final conc.	Vol. for 10 mL
0.5 M EDTA pH 8.0	50 mM	1 mL
1 M Tris HCl, pH 8.0	100 mM	1 mL
MilliQ H_2O	--	8 mL
15 µg/mL Proteinase K	3 ng/mL	2 µL

Achromopeptidase permeabilization solution (60 U/mL final)

Stock	Final conc.	Vol. for 2 mL
5 M NaCl	10 mM	4 µL
1 M Tris HCl, pH 8.0	100 mM	20 µL
MilliQ H_2O	--	1.97 mL
Achromopeptidase (30K U/mL)	60 U/mL	4 µL

Hybridization buffer

1. Add the appropriate volume of MilliQ H_2O, NaCl, and Tris-HCl to a 50 mL falcon tube.
2. Add 2 g of dextran sulfate, heat to 40-60°C, and shake until the dextran sulfate has dissolved completely.
3. Cool to ~room temperature, add the appropriate volume of formamide, blocking reagent, and SDS, and aliquot in 10 mL volumes in 15 mL Falcon tubes. Frozen stocks can be stored at -20°C for several months.

Stock	Final conc.	Vol. for 20 mL
5 M NaCl	900 mM	3.6 mL

1 M Tris HCl, pH 8.0	20 mM	0.4 mL
MilliQ H_2O	--	(see below)
Blocking reagent, 10% stock*	1%	2.0 mL
Dextran sulfate	0.1 g/mL	2.0 g
100% Formamide	--	(see below)
10% SDS	0.02%	40 µL

*Blocking Reagent (Roche, cat#11096176001) should be prepared as a 10% stock in maleic acid buffer according to the manufacturer's instructions. Stock aliquots can be stored at -20°C.

Wash buffer (can be made in advance)

Stock	**Final conc.**	**Vol. for 50 mL**
0.5 M EDTA pH 8.0	5 mM	0.5 mL
1 M Tris HCl, pH 8.0	20 mM	1 mL
5 M NaCl	--	(see below)
MilliQ H_2O	--	(see below)
10% SDS	0.01%	50 µL

Volumes of formamide, NaCl and water for hybridization and wash buffers of varying stringencies, for hyb at 35°C and wash at 37°C

	Hyb buffer (20 mL)			**Wash buffer (50 mL)**	
%Form in Hyb Buffer	**mL Form**	**mL H_2O**		**mL 5M Nacl**	**mL H_2O**
0	0	14			
5	1	13			
10	2	12			
15	3	11			
20	4	10		1.350 (0.145 M)	47.10
25	5	9		0.950 (0.105 M)	47.50
30	6	8		0.640 (0.074 M)	47.81
35	7	7		0.420 (0.052 M)	48.03
40	8	6		0.270 (0.037 M)	48.18
45	9	5		0.160 (0.026 M)	48.29

50	10	4		0.090 (0.019 M)	48.36
55	11	3		0.030 (0.013 M)	48.42
60	12	2		0 (0.009 M)	48.45
65	13	1		0 (0.008 M)	48.45
70	14	0		0 (0.005 M)	48.45

Mounting solution (make fresh)

Stock	Final conc.	Vol. for 2 mL
50 µg/mL DAPI	2 µg/mL	80 µL
1x PBS	0.5	140 µL
Vectashield	1	280 µL
Citifluor	5.5	1540 µL

Stock storage

Stock	Storage	Notes
5M NaCl	RT	
0.5M EDTA, pH 8.0	RT	
1.0M Tris HCl pH 8.0	RT	
SDS, 10% (w/v)	RT	
0.01M HCl	RT	
10x PBS	RT	
100 mg/mL lysozyme	-20°C	Store as 1 mL aliquots
15 µg/mL Proteinase K	RT	
Achromopeptidase (30K U/mL)	-20°C	
10% blocking reagent	-20°C	
100% formamide	RT	
Hybridization buffer	-20°C	Store as 10 mL aliquots
Wash buffer	RT	Fresh filter prior to use
50 µg/mL DAPI	-20°C	Store as 1 mL aliquots
Vectashield	4°C	
Citifluor	RT	

Appendix 2. Product list: reagents and supplies

Product	Company	Catalog #	Quantity	Vendor
EM grade paraformaldehyde, 20%	Electron Microscopy Sciences	15713	10x 10 mL	VWR
0.1 µm pore sized, 25 mm diameter polycarbonate membranes; white	GE Osmonics	K01CP02500	100/pkg	Fisher
GN-4 Metricel membranes, 25 mm diameter	Pall Corp.	66263	100/pkg	VWR
Phosphate buffered saline (PBS), 10x solution	Fisher	BP399-1	1 L	Fisher
Vacuum Dessicators	VWR	62344-926	600 mL	VWR
Indicator dessicant	VWR	61161-319	40/pkg	VWR
Falcon Disposalbe Petri dishes, 35 x 10 mm	Corning	351008	500/case	VWR
Falcon 6-well cell culture plate	Corning	351146	50/case	VWR
SeaPlaque GTG agarose	Lonza	50111	25 g	VWR
Ethanol, 190 proof	Sigma	E7148-6X500ML	6x 500 mL	Sigma
Plain microscope slides, 75x50 mm	Corning	2947-75X50	720/case	VWR
Gel blotting paper, 15 x 20 cm	Whatman	10427812	100/pkg	VWR
Lysozyme, egg white, OmniPur	EMD Millipore	5960-10GM	10 g	VWR
Proteinase K, >600 mAU/mL	Qiagen	19131	2 mL	Qiagen
Achromopeptidase	Sigma	A3547-100KU	100,000 U	Sigma
Formamide	Sigma	47671-250ML-F	250 mL	Sigma
Dextran sulfate	Sigma	D8906-50G	50 g	Sigma
Blocking Reagent	Roche	11096176001	50 g	Roche
Maleic acid	Sigma	M0375-500G	500 g	Sigma
TSA Plus Cyanine 3 System	Perkin Elmer	NEL744001KT	Ea.	Perkin Elmer
Citifluor AF1	Citifluor Ltd.	17970-25	25 mL	VWR
DAPI	Life Technologies	D1306	10 mg	Life
Vectashield Mounting Medium	Vector Laboratories	H-1000	10 mL	VWR

Misc. not listed:

Filtration manifold, filter towers, vacuum pump, vacuum trap, 20, 200, 1000, and 5000 µL pipettes with tips, 10, 25, and 50 mL disposable Pasteur pipettes, foreceps, razor blades or X-acto knives, parafilm, slide boxes, gallon ziplock freezer bags, aluminum

foil, hybridization oven with hyb tubes, microwave oven, 1.5 mL microcentrifuge tubes (amber), 50 and 15 mL Falcon tubes, microscope slides, cover slips, fingernail polish, immersion oil, micrometer for measuring field of view, amber or foil covered polypropylene bottles of appropriate size for fixing samples.

Appendix 3. Rough estimate of volumes to filter by depth at Station ALOHA

Depth (m)	Est. cells/mL (heterotrophs)	Vol. for 100 cells/FOV	Rounded	Vol. 20%PFA	Total vol. to filter
0-10	4.4×10^5				
11-25	4.7×10^5				
26-50	4.7×10^5				
51-75	4.2×10^5				
76-100	3.9×10^5				
101-125	3.1×10^5				
126-150	2.6×10^5				
151-175	2.2×10^5				
176-200	2.0×10^5				
201-300	1.5×10^5				
450-500	6.4×10^4				
750	4.3×10^4				
1000	4.2×10^4				

Appendix 4. Miscellaneous Useful Information

Fixation

- Decrease protein solubility and stop all cellular activity. Often achieved by the use of aldehydes or alcohols.
- For FISH, formaldehyde is most often the fixative of choice – low autofluorescence.
- Formalin = 37% formaldehyde solution. Best results achieved with making your own formaldehyde stock solution from paraformaldehyde powder (e.g. 10% or 20% stock), or using electron microscopy grade formaldehyde.
- Most often used at final concentrations from 1-4%.
- Always freshly filter PFA solution prior to each use (not needed for fresh EM grade). Do not store for extended periods at 4°C.

Filtration

- Important to filter a volume of sample that will yield an appropriate density of cells for enumeration.

  vol. to filter = (# cells/grid or FOV) x (1/grid area) x (filter area) x (1/cells per mL)

Need to know: area of grid or field of view (xxxx μm^2)
functional area of membrane (yyyy μm^2)
estimate of cellular abundance in sample

- Equation above is simply a rearrangement of:

  cells/mL = (# cells/grid) x (1/grid area) x (filter area) x (1/vol filtered)

- Filter under low pressure; filter brand and color is personal preference, or what looks best under your microscope. My preference is Osmonics 0.1 µm pore-sized polycarbonate membranes for FISH. Backing membrane: GN4 (mixed cellulose)

Hybridization

- Target cells are incubated in the presence of excess probe molecules. Nucleation reaction thought to be limiting step, which is a second order kinetic rxn. Therefore, the higher the probe concentration, the higher the reannealing rate.
- Requires an aqueous environment.
- Works well if cells are re-hydrated with hybridization solution containing probe (that's one reason to keep the membrane filters dry).
- Incubation occurs at the melting temperature of the oligo/target hybrid: incubation close to the melting temperature increases specificity (i.e. ability to discriminate 100% matching target from non-identical target sites).

Washing

- Goal is to remove excess probe and ensure that oligonucleotide is bound only to specific target sequence.
- Stringency is achieved by washing close to the melting temperature of the oligonucleotide-target hybrid.
- Melting temperature (T_m): point at which 50% of homoduplexes between oligonucleotide and target are denatured - midway point between single and double stranded conformations.

Melting temperature (T_m)

- Theoretical equations are inaccurate, but still provide a useful estimate to begin empirical testing. The actual wash temperature should be determined empirically (i.e. hybridizing at a range of formamide concentrations and corresponding salt concentrations in the wash, or a range of temperatures).
- This can be performed for isolates (ideally) or environmental samples, but requires a means to quantify fluorescence per cell.
- What affects melting temperature?

  Length and base composition of oligonucleotide
  Chemical composition of solution

- Most common equation for calculating T_m for DNA-RNA hybrids:

  $T_m = 79.8 + 18.5(\log_{10}M) + 58.4(\%GC) + 11.8(\%GC)^2 - 820/L - 0.63(\%F)$

  M = concentration of monovalent cations; usually just [Na+]
  %GC = <u>fractional</u> percent GC of the oligonucleotide probe
  L = length of the oligonucleotide
  %F = % formamide

- Thus:
  Increasing salt concentration increases the T_m
  A longer oligonucleotide probe increases the T_m
  Higher %GC of probe increases the T_m
  Addition of formamide decreases the T_m
- ***So***, for a given oligo, stringency can be achieved by increasing temperature, decreasing salt, or increasing formamide.
  Salts: Monovalent cations interact electrostatically with nucleic acids by decreasing the electrostatic repulsion between the two strands. Higher concentrations thus increase the stability of the hybrid.
  Formamide: organic solvent that reduces the stability of double stranded DNA by interfering with H-bonding. However, it also reduces the nucleation-rate-constant for reassociation, effectively slowing the reaction and necessitating longer hybridization conditions.
  Most hybridizations will remove the formamide from the wash buffer and adjust stringency by salt or temperature (slow stringent reaction for the hybridization, fast stringent reaction for the wash).

- **In the real world, melting temperature for oligos can be estimated by:**
  $T_m = 4(\#GC) + 2(\#AT) + 8°C$
  This holds true for [Na+] = 0.9M and 0% Formamide

Example calculation to estimate formamide concentration in the hybridization buffer for a hyb reaction at 35°C.
Example oligo: 20-mer, 8GC and 12AT (40% GC).
Quick T_m estimate = $4(\#GC) + 2(\#AT) + 8°C = 64°C$
More precise $T_m = 79.8 + 18.5(\log_{10}M) + 58.4(\%GC) + 11.8(\%GC)^2 - 820/L - 0.63(\%F)$
In standard hyb buffer with no formamide and 0.9M NaCl, the calculated T_m is:
$= 79.8 + (18.5*-0.0457575) + (58.4*0.4) + (11.8*0.16) -820/20 = 64.9°C$
So, to hybridize at 35°C, the calculation would be:
$35°C = 65°C -0.63(\%F)$, or $\%F = (35 -65)/-0.63 = 60\%$

General reference regarding FISH: http://www.arb-silva.de/fish-probes/

Appendix 5. Random citations

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