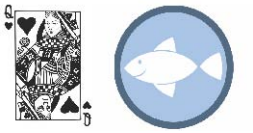
CARD-FISH and Microautoradiography

Protocol for Bacteria and Archaea



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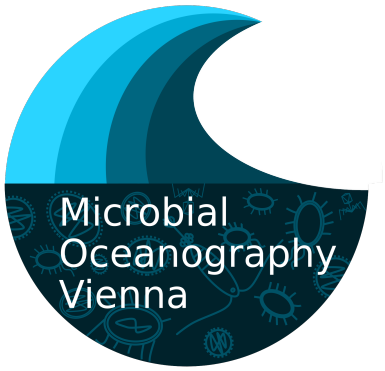

Sample Fixation

1. Fix samples with 0.2 µm filtered 37% formaldehyde to final concentration of 2-4%. After fixation, store at 4ºC for at least 1h but not longer than 24h.
2. Filtrate sample on white polycarbonate filter placing on cellulose nitrate support filter. Check the abundance of cells in samples to estimate the volume to filter. For example:

Abundance of cells in sample	Volume to filter (ml)
$1x10^4$ cell ml^{-1}	50 (minimum)
$5x10^5$ cell ml^{-1}	10
$1x10^6$ cell ml^{-1}	5

3. After sample filtration, wash twice with 5-10 ml of MQ.
4. Air-dry filters
5. Store at –20ºC until processing. Filters can be stored frozen for several months.

Embedding

1. Boil 0.1% Agarose in a microwave and cool down to 35-40ºC. Pipette 50-55 µl of the Agarose on petri dish. Dip filter with both sides in Agarose and place filters sample side down on drop.
2. Let filters dry at 37ºC in hybr. oven without lid for 10-15min.
3. Pour ethanol (96%) in petri dish and carefully remove filters.
4. Air-dry filters, now they can be stored at -20°C.

Permeabilization

1. Cut filters (e.g. 1/12 size of a filter for a hybridization).
2. **Bacteria**: Prepare 10 ml of permeabilization mix with lysozyme (see below).

Stock reagent	Volume (µl)	Final
Lysozyme	100 mg	10 mg/ml
1M Tris-HCl	1000	0.1 M
0.5M EDTA	1000	0.05 M
MQ water	8000	

* after preparation put it on ice.

3. **Bacteria**: Pour lysozyme mix in petri dish and place filters sample side down into it, incubate for 1h at 37ºC in hybr. oven.
   After 1h, wash filters 1x in excess MQ.
   Archaea: For permeabilization of Archaea, incubate filters in 0.1M HCl for 1 min at room temperature (RT) (Woebken et al. 2007). Afterwards wash filters 3x in excess MQ.
4. Place all filters for 20-25min in 0.01M HCl at RT to inactivate endogenous peroxidases.
5. Wash filters 2x in excess MQ
6. Shortly dip them into Ethanol (96%)
7. Dry filters, now they can be stored at -20 ºC.

Hybridization

1. Mix hybridization buffer with HRP probe (final volume 300 µl → see p. 6). Freeze probes just once, after thawing store them at 4ºC for up 0.5 year.
2. Place filter sections into 0.6 ml Eppi. You can put multiple 1/12 size filter sections maximum ca. 20 filters/ tube.
3. Hybridize at 35ºC for 12-15h in hybrid. oven. in the dark. Stick Eppis on rotor and shake slowly.

Washing

1. Prepare washing buffer (final volume 50 ml) and pre-warm it in a 37ºC water bath.
2. The volume of 5M NaCl in the Washing Buffer varies between probes (see p. 5). Here are examples:

Bacteria - Washing Buffer for 35ºC hybr. temp. and 55% Formamide in hybridization buffer:

Stock reagent	Volume (µl)	Final
5M NaCl	30*	13mM
1M Tris-HCl	1000	20mM
0.5M EDTA	500	5mM
MQ water	48420	
10% SDS	50	0.01%

Archaea - Washing Buffer for 35ºC hybr. temp. and 20% Formamide in Hybridization buffer:

Stock reagent	Volume (µl)	Final
5M NaCl	1350*	145mM
1M Tris-HCl	1000	20mM
0.5M EDTA	500	5mM
MQ water	47100	
10% SDS	50	0.01%

3. After hybridization, quickly transfer filter sections into washing buffer under fume hood.
4. Keep filters in washing buffer for 15 min at 37ºC waterbath, afterwards pour in to buechner funnel.

Amplification

1. Prepare PBS-T-Mix:

Stock reagent	Volume	Final
1xPBS	50 ml	
100% Triton X100	25 µl	0.05%

PBS should have pH of 7.4 -7.6

2. Pick filters and incubate in 25 ml PBS-T-Mix at RT for 10-15min in the dark.
3. Prepare substrate with mixing prepared amplification buffer (AMP), 30% H_2O_2, and Alexa Tyramide. Use 1.5 ml Eppis (A, B) to dilute 30% H_2O_2 to final concentration of 0.0015% in Eppi B.

	AMP (µl)	H_2O_2 (µl)	Tyr (µl)	Ratio
A	200	1 from 30%		
B	493	5 from A	5	1:100

4. Dab filters on blotting paper, place sections on top of each other. Place in Eppi B with the substrate mix.
5. Incubate it for 15min at 46ºC in hybrid. oven in the dark.
6. After incubation, dab filters on tissue paper to remove excess substrate mix.
7. Wash in 25ml PBS-T mix at RT for 15min in the dark.
8. Wash 3x in excess MQ
9. Shortly dip them into Ethanol (96%).
10. Dry and mount in DAPI mix or store at -20 ºC for further experiments (MICRO-CARD-FISH).

According to Pernthaler et al. (2002) with some modifications

Hybridization Buffers - 10ml:
Store at -20ºC for up to 1 year; when in use keep on ice!
For the different probes, check p.7-8 for information of formamide concentration.

Stock reagent	Volume (µl)	Final conc.
5M NaCl	1800	900 mM
1M Tris-HCl	200	20 mM
Dextran Sulfate	1 g	10%
100% Triton X100	5	0.05%

Bring in solution at 40-60ºC in water bath, takes about 30min then cool down on ice. Then add:

Formamide final conc %	60	55	45	40	35	20
Formamide (ml)	6.0	5.5	4.5	4.0	3.5	2.0
10% Blocking (ml)	1					
Sigma Water (ml)	1.0	1.5	2.5	3.0	3.5	5.0

Amplification Buffer (0.1% blocking) - 20 ml:

Stock reagent	Volume (µl)	Final conc.
Dextran Sulfate	2g	10%
5M NaCl	8000	2 M
10% Blocking	200	0.1%
1x PBS	11800	

* Store at 4°C in 50 ml Greiner tube.

Amplification Buffer (NO Blocking)* - 9 ml:

Stock reagent	Volume (µl)	Final conc.
Dextran Sulfate	1g	>10%
5M NaCl	4000	>2M
1x PBS	5000	

*If needed. Store at 4°C in 50 ml Greiner tube.

Amplification buffer (0.5, 1.0% Blocking)*-0.7 ml:

Stock reagent (µl)	AMP 1% Blocking	AMP 0.5 % Blocking
AMP (No Blocking)	630	630
10% Blocking reagent	70	35
1x PBS	0	35

* If needed.

DAPI 50 µg/ml – 500 µl:

Stock reagent	Volume (µl)	Final
DAPI 1 mg/ml	25	50 µg/ml
1xPBS	475	

DAPI mix 2 µg/ml - 1 ml:

Stock reagent	Volume (µl)	Final
DAPI 50µg/ml	40	2 µg/ml
1xPBS	70	0.5
Vectashield	140	1
Cititfluor	750	5.5

6 N HCl - 100 mL:

- MQ 30 mL
- ↓ ← HCl conc. 50 mL
  ↓
- Fill up to 100 mL with MQ (keep in a plastic or a glass bottle)
  * Do not autoclave. HCl conc. includes 35-37% of HCl which is experientially considered 12 M (= 12 N for HCl).

5 N NaOH - 100 mL:

- MQ 80 mL
- ↓ ← NaOH (sodium hydroxide, solid, MW: 40.00) 20 g
  ↓
- Fill up to 100 mL (keep in a plastic bottle).
  *Do not autoclave.

0.5 M EDTA - 500 mL:

*For what: to reduce the effect of heavy metal and inactivate enzymes with adding buffer or enzymatic reaction reagent. Use 0.1-1mM concentration and pH=7.0-8.0. Chelate reagent.

Chemicals needed

- EDTA2Na-2H_2O (Ethylenediaminetetraacetic acid disodium salt dihydrate, MW:372.24)
- NaOH (pastilles)
- 5 N NaOH

MQ 400 mL
↓ ← EDTApowder 93.06 g

Mix and measure pH
↓ ← NaOH (pastilles) ca. 10 g

Adjust to pH 8.0 with 5 N NaOH. EDTA will dissolve only at pH 8!
↓
Fill up to 500 mL
↓
Autoclave
↓
Keep at RT or in fridge

10x PBS - 500 mL:

*For what: representative for saline solution. To wash and suspend cells.

Chemicals needed

- NaH_2PO_4 (MW: 119.98, acid) 1.38 g
- Na_2HPO_4 (MW: 141.96, alkaline) 7.12 g
- NaCl 37.985 g

MQ 400 mL
↓ ←Chemicals listed above

Adjust to pH 7.6 with 6N HCl or 5N NaOH
↓
Fill up to 500 mL
↓
Autoclave
↓
Keep at RT or in fridge

1x PBS - 1000 mL:

10x PBS 100 mL
MQ 850 mL
↓
Mix and adjust to pH 7.6
↓
Fill up to 1000 mL
↓
Autoclave

1 M Tris-HCl buffer - 500 mL:

*For what: common buffer for biological lab work proper working pH=7.1-8.0.

Chemicals needed

- Tris-hydroxymethyl-aminomethane, MW: 121.14
- 6N HCl

MQ 400 mL
↓ ←Tris powder 60.55 g

Adjust to pH 8.0 with 6 N HCl
(approx. 50 mL of 6 N HCl)
↓
Fill up to 500 mL
↓
Autoclave
↓
Keep it at RT or in fridge

5 M NaCl - 250 mL:

MQ 200 mL
↓ ←NaCl (MW: 58.44) 73.05 g

Fill up to 250 mL
↓
Autoclave
↓
Keep it at RT or in fridge

Maleic acid buffer - 50 mL:

Chemicals needed

- Maleic acid (MW: 116.08) 0.580 g
- NaCl (MW:58.44) 0.438 g

MQ 35 mL in a 50-100 mL glass screw cap bottle
↓ ←Chemicals listed above

Adjust to pH 7.5 with NaOH (ca. 2 solid tablets plus 5 N NaOH)
↓
Fill up to 50 mL

Blocking Reagent 10% - 50 mL:

Chemicals needed

- Boehringer Mannheim Blocking reagent (check expire date)

50 mL of Maleic acid buffer (mentioned above)
↓ ←Blocking reagent 5 g

Dissolve reagent on heating plate at 60 ºC with stirring for about 1h. Do not boil.
↓
Autoclave
↓
Make 5 mL aliquots. Pipette carefully (800 µL is max for 1mL pipette tip, as it is viscous liquid).
↓
Keep at -20ºC. According to the manufacture solution should be good for a few days at 4ºC and many months if stored frozen.

Tyramide Synthesis

Chemicals needed

- 1 mg A_{488} Succinimidyl ester
  and/or
- 1 mg A_{555} Succinimidyl ester

- N,N-Dimethylformamide (waterfree)
- Tyramine-HCL
- Triethylamine

Abbreviations used

DMF: N,N-Dimethylformamide
F_W=73.09 g/mol
TEA: Triethylamine
F_W=101.19 g/mol
TYR-HCL: Tyramine-HCL
M_w = 173.64 g/mol

Solutions

1. **DMF-TEA stock:**
   1ml DMF + 10 µl TEA
   Prepare in 2 ml Eppi
2. **TYR Stock:**
   10 mg TYR-HCL + 1 ml DMF-TEA stock
   100 µl TYR.stock = 5.76 µmol/l
   Prepare in 15 ml Greyner

Triethylamine & Dimethylformamide are dangerous: work in the hood and on ice!

3. **Succinimidyl ester:**
   1mg active ester + 100 µl DMF
   1 mg $Alexa_{488}$ = 1.6 µmol/l

   $Alexa_{488}$: M_w = 643.41 g/mol
   λ_{max} = 495 nm
   E_{max} = 519 nm
   ε = 71,000
   C_f = 0.11

   $Alexa_{555}$: M_w = 1250g /mol
   λ_{max} = 555 nm
   E_{max} = 565 nm
   ε = 150,000
   CF_{280} = 0.08

Esters are light sensitive (cover with aluminum foil) and prone to hydrolysis, therefore prepare shortly before tyramide synthesis; cool esters on ice until they are used for synthesis.

Synthesis

1. 100 µl $Alexa_{488}$ + 25.2 µl TYR stock
   100 µl $Alexa_{555}$ + 12.6 µl TYR stock
2. Incubate at room temperature in the dark for ~12 hours, slowly rotating
3. Dilute to 1 ml with absolute Ethanol (874.8 µl for A_{488} and 887.4 µl for A_{555})
4. Make aliquots (~50 µl) and store at -20°C.

Washing Buffers (FISH protocols in Silva)

NaCl concentration in the washing buffer for washing at 37°C after hybridization at 35°C

% formamide in hybridization buffer	Final conc. of NaCl (M)	5 M NaCl (µl) in 50 ml of washing buffer
20	0.145	1350
25	0.105	950
30	0.074	640
35	0.052	420
40	0.037	270
45	0.026	160
50	0.019	90
55	0.013	30
60	0.009	0
65	0.008	0
70	0.005	0

NaCl concentration in the washing buffer for washing at 48°C after hybridization at 46°C

% formamide in hybridization buffer	Final conc. of NaCl (M)	5 M NaCl (µl) in 50 ml of washing buffer
0	0.900	9000
5	0.636	6300
10	0.450	4500
15	0.318	3180
20	0.225	2150
25	0.159	1490
30	0.112	1020
35	0.080	700
40	0.056	460
45	0.040	300
50	0.028	180
55	0.020	100
60	0.014	40
65	-	-
70	-	-

Measure concentration of DNA (Wendeberg 2010)

Ordered probe (dry), Biomers
↓ ←xx µl* of Sigma water
Make 100 pmol/µL
↓
Dilute x1/2 (x1/4) with Sigma water (ex. 1µL of probe + 1µL of Sigma water).
↓
Measure DNA concentration (260 and 404 nm) with Nanodrop 2000.

*Volume is written on the product information sheet from Biomers. Normally it's 50-100 µl, when you order Scale "S".

In our case (UV-Vis application, 1mm path length),
OD_{260} = Abs 260 nm ÷ 0.1 cm
OD_{404} = Abs 404 nm ÷ 0.1 cm

Calculate the concentration of the single-stranded DNA oligonucleotide present in the sample*:
$OD260_{adj} = OD_{260} - (OD_{404} \times 0.276)$
DNA concentration (ng/µl) = $OD260_{adj} \times 20$

*Both HRP and the probe contribute to the absorption maximum 260 nm. The measured concentration therefor must be decreased by a correction factor of 0.276.

Labelling efficiency

Presuming optimal labeling, the peak ratio (OD_{260}/OD_{404}) should be ~3.

Make aliquots

Dilute the probe to make 50 ng/µl with adding Sigma water
↓
Make 20-50 µl of aliquots in UV sterile 0.6 mL tubes.
↓
Keep them at -20ºC

Volume of probes to add into hybrid. buff.

In our lab, currently there are three different concentrations of probe working solutions prepared. Depending on the DNA concentration, amounts need to be added to the Hybridization Buffer is different (shown in box below). The final volume of Hybridization Buffer + probe(s) is 300 µl.

Company	Conc. in stock	Vol (µl)
Biomers	50 ng/µl of DNA	1.0
Thermo (10x)	500 ng/µl of DNA+HRP	1.5
Thermo (1x)	50 ng/µl of DNA+HRP	15.0

*Approx. vol. The best concentration of probe is depending on sample.

4. CARD FISH –probe information–

List of oligonucleotide probes targeted to marine prokaryotes. Probes and hybridization conditions currently used in Microbial Oceanography Lab is shown in bold.

***Hybridization temperature: 35 ºC, washing temperature: 37 °C**

Target organisms	Probe			Hybridization condition	
	Name	Sequence	Ref	Formamide conc. in hybrid. buffer (%)	Ref.
Bacteria	EUB338 I	GCT GCC TCC CGT AGG AGT	Amann et al. (1990)	55	Pernthaler et al. (2002)
	EUB338 I **EUB338 II** **EUB338 III**	**GCT GCC TCC CGT AGG AGT** **GCA GCC ACC CGT AGG TGT** **GCT GCC ACC CGT AGG TGT**	Amann et al. (1990) Daims et al. (1999) * suppliment to EUB338I Daims et al. (1999) * suppliment to EUB338I	**55**	Ishii et al. (2004)
Thaumarchaeota	Cren554	TTA GGC CCA ATA ATC MTC CT	Massana et al. (1997)	20	Pernthaler and Amann (2005)
	Cren537	TGA CCA CTT GAG GTG CTG	Teira et al. (2004)	20	Teira et al. (2004)
	Cren554 **Cren537**	**TTA GGC CCA ATA ATC MTC CT** **TGA CCA CTT GAG GTG CTG**	Massana et al. (1997) Teira et al. (2004)	**20**	Lekunberri et al. (2013)
Euryarchaeota	**Eury806**	**CAC AGC GTT TAC ACC TAG**	Teira et al. (2004)	**20**	Teira et al. (2004)
Negative control	**NON338**	**ACT CCT ACG GGA GGC AGC**	Wallner et al. (1993)	**55**	Pernthaler et al. (2002)
SAR11 clade	SAR11-152R SAR11-441R SAR11-542R SAR11-732R	ATT AGC ACA AGT TTC CYC GTG T TAC AGT CAT TTT CTT CCC CGA C TCC GAA CTA CGC TAG GTC GTC AGT AAT GAT CCA GAA AGY TG	Morris et al. (2002)	15	Morris et al. (2002) *FISH, 37ºC hybrid temp
	SAR11-152R SAR11-441R SAR11-542R SAR11-732R	ATT AGC ACA AGT TTC CYC GTG T TAC AGT CAT TTT CTT CCC CGA C TCC GAA CTA CGC TAG GTC GTC AGT AAT GAT CCA GAA AGY TG	Morris et al. (2002)	45	Lekunberri et al. (2013)
	SAR11-441R	TAC AGT CAT TTT CTT CCC CGA C	Morris et al. (2002)	50	Mary et al. (2006)
	SAR11-441R	**TAC AGT CAT TTT CTT CCC CGA C**	Morris et al. (2002)	**45**	Pernthaler and Amann (2005)
	SAR11-486	GGA CCT TCT TAT TCG GGT	Fuchs et al. (2005)	60	Fuchs et al. (2005)
SAR202 clade	SAR202-104 SAR202-312	GTT ACT CAG CCG TCT GCC TGT CTC AGT CCC CCT CTG	Morris et al. (2004)	35	Morris et al. (2004) *FISH, 37ºC hybrid temp
	SAR202-312	TGT CTC AGT CCC CCT CTG	Morris et al. (2004)		
SAR324 clade	SAR324-1412	GCC CCT GTC AAC TCC CAT	Schattenhofer et al. (2009)		
SAR406 clade	SR406-97	CAC CCG TTC GCC AGT TTA	Fuchs et al. (2005)	65	Fuchs et al. (2005)
Alteromonas /Colwellia	Alt1413	TTT GCA TCC CAC TCC CAT	Eilers et al. (2000)	55	Lekunberri et al. (2013)
	Alt1413	TTT GCA TCC CAC TCC CAT	Eilers et al. (2000)	50	Pernthaler and Amann (2005)

*Please double check the sequence when you order.

4. CARD FISH –probe information–

***Hybridization temperature: 46 °C, washing temperature: 48 °C**

Target organisms	Probe			Hybridization condition	
	Name	Sequence	Ref.	Formamide conc. in hybrid. buffer (%)	Ref.
Bacteria	EUB338 I	GCT GCC TCC CGT AGG AGT	Amann et al. (1990)	35	
	EUB338 II	GCA GCC ACC CGT AGG TGT	Daims et al. (1999) * suppliment to EUB338I		
	EUB338 III	GCT GCC ACC CGT AGG TGT	Daims et al. (1999) * suppliment to EUB338I		
Thaumarchaeota	Cren554	TTA GGC CCA ATA ATC MTC CT	Massana et al. (1997)	0	
	Cren554	TTA GGC CCA ATA ATC MTC CT	Massana et al. (1997)		
	Cren537	TGA CCA CTT GAG GTG CTG	Teira et al. (2004)		
Euryarchaeota	Eury806	CAC AGC GTT TAC ACC TAG	Teira et al. (2004)	0	
Negative control	NON338	ACT CCT ACG GGA GGC AGC	Wallner et al. (1993)	35	
SAR11 clade	SAR11-486	GGA CCT TCT TAT TCG GGT	Fuchs et al. (2005)	25	Schattenhofer et al. (2009)
	SAR11-152R	ATT AGC ACA AGT TTC CYC GTG T	Morris et al. (2002)	25	Gomez-Pereira et al. (2013)
	SAR11-441R	TAC AGT CAT TTT CTT CCC CGA C			
	SAR11-441R (modif)	TAC CGT CAT TTT CTT CCC CGA C	Gomez-Pereira et al. (2013)		
	SAR11-487 (modif)	CGG ACC TTC TTA TTC GGG			
	SAR11-487-H3	CGG CTG CTG GCA CGA AGT TAG C			
	SAR11-542R	TCC GAA CTA CGC TAG GTC	Morris et al. (2002)		
	SAR11-732R	GTC AGT AAT GAT CCA GAA AGY TG			
	SAR11-486	GGA CCT TCT TAT TCG GGT	Fuchs et al. (2005)	25	Teeling et al. (2016)
	SAR11-441R	TAC AGT CAT TTT CTT CCC CGA C	Morris et al. (2002)		
SAR202 clade	**SAR202-312**	**TGT CTC AGT CCC CCT CTG**	Morris et al. (2004)	**40**	Schattenhofer et al. (2009)
SAR324 clade	SAR324-1412	GCC CCT GTC AAC TCC CAT	Schattenhofer et al. (2009)	35	Schattenhofer et al. (2009)
SAR406 clade	**SAR406-97**	**CAC CCG TTC GCC AGT TTA**	Fuchs et al. (2005)	**40**	Schattenhofer et al. (2009)
Alteromonas /Colwellia	**Alt1413**	**TTT GCA TCC CAC TCC CAT**	Eilers et al. (2000)	**40**	Eilers et al. (2000)

*Please double check the sequence when you order.

5. MICROAUTORADIORAPHY

Cell sampling and fixing

1. Check the abundance of cells in your samples, to estimate the volume to filter (see p. 2).
2. Transfer samples in Greiner tubes (samples and killed controls). Fix controls with 0.2 µm filtered formaldehyde (final conc. 2-4%) and wait for 15min.
3. Add radioactive labeled compounds for both samples and killed controls, e.g.:

Substrate	Final concentration in sample*
^{3}H-Leucine	40 nM (coastal) 10-20 nM (epipelagic) 5 nM (meso-, bathypelagic)
^{3}H-Thymidine	20 nM
^{14}C-HCO_3^-	50-100µCi

*Above concentrations are for marine systems. They are highly depending on sample.

4. Incubate for at least 4h in the dark under in situ temperature. Take into account the expected level of activity for your sample and the substrate activity (e.g. for deep sea: 24h for ^{3}H-leucine, 72h for ^{14}C-bicarbonate).
5. Kill all samples with 0.2 µm filtered formaldehyde (final conc. 2-4%) and incubate at 4ºC in the dark ~24h.
6. Filter the samples and store them (see p. 2).

Transfer of filter sections

1. Place the sample filter sections on slides covered with plastic foam sheets (samples side up). If the section size is 1/12 of a filter, 8-10 pieces are possible to be placed on a slide. This process can be done while melting the emulsion (next step).

   All the following steps must be performed in the dark with a safety lamp, although complete darkness ensures a better result!

2. Melt the emulsion prepared in the tube (2 slide mailer) for 0.5-1h at 43ºC in a water bath in the dark.
3. Coat the slides with the prepared emulsion by dipping them into the tube.
4. Remove the emulsion from the underside of the slide with clean tissue paper.
5. Dry the slides for 5-10 min (the time it takes to process 4 slides) on ice cold aluminum plates. This is best done in a small tray containing ice covered with aluminum foil.
6. One by one, take the prepared filter sections and carefully mount them on the slides. Sample filter sections are transferred to the emulsion slides.
7. Place the slides in a light proof box wrapped twice with aluminum foil, containing silica gel drying beads, for exposure* at 2-4ºC.
   *The exposure time is dependent on the activity of the substrate and expected activity of the cells.

Developing and Fixing

The Dark room should be at around 17ºC. All developing chemicals and MQ should be kept in at 17ºC.

1. Place box with slides in the dark room (17°C) for 1h before starting.
2. Prepare developing chemicals in slide staining jars.

   All the following steps must be performed in complete darkness!

3. Remove aluminum foil and place the slides in the slide rack.
4. **Kodak:**
   Develop the slides using Kodak specifications: 2 min in Dektol Developer, 10s to stop developing in MQ, 5 min in Kodak Fixer, 2 min in MQ.
   Ilford:
   Develop the slides using Ilford specifications: 4 min in Ilford Phenisol Developer, 10s to stop developing in MQ, 6-7 min* in Ilford Hypam Fixer, 5 min in MQ.
   *According to the manufacture's protocol, 4 min is recommended. If the emulsion is not clear enough within 4 min, fixing the material for twice the time is also applicable. Therefore, we currently chose 6-7 min for fixing.

   After this you can turn on the light!

5. Keep the slides in the MQ until you are back in the lab. Dry the underside of the slide
6. Surround the borders of the filters on the underside of the slide with a marker. This is also the last chance to know from the filter labels which section is which - so label the slide accordingly.
7. Dry the side with filters with tissue. Remove only the outer edge of the filter section and dry for 10-20 min.
8. Carefully peel the filter sections off. If it is difficult to hold the filter with forceps, make the edge of filter wet again with wetted tissue paper.
9. Put a drop of DAPI-mix in each filter outline and put a cover slip over it.
10. They can be stored at -20ºC until they are counted on the epifluorescence microscope.

6. MICROAUTORADIOGRAPHY –Chemicals and Buffers–

Emulsion (Kodak and Ilford)

Kodak:

The Emulsion is a mix of Kodak NTB and Sigma water 2:1 (v/v) = 7 ml + 3.5 ml

Ilford:

The Emulsion is a mix of Ilford Nuclear Emulsion Type K5 and Sigma water 1:1 (v/v) = 5 ml + 5 ml

Take into account the emulsion has an expiration date of few months. Each tube with the emulsion can be used a max. of three times in total ca. 15 slides.

1. Melt the emulsion for 1 h at 43°C before use.
2. Wrap the 2 slide mailer (tubes) with Aluminium tape (or Aluminium foil plus duct tape) and ensure that the lid is covered with black marker pen.
3. Add 3.5 ml (Kodak) or 5 ml (Ilford) of Sigma water in the tubes.
4. In the completely dark condition, add 7 ml (Kodak) or 5 ml (Ilford) of photographic emulsion with a dispenser. The emulsion is highly sensitive to light; therefore, complete darkness is recommended. This is best done using a bottle top dispenser.
5. Wrap the tubes in aluminum foil twice and store at 4°C.

Kodak Fixer (250 ml)

Chemicals needed

- Kodak Fixer

MQ 198 mL
↓ ←Fixer powder 46 g
Mix and fill up to 250 mL
↓
Keep it at 17ºC

Kodak Developer (250 ml)

Chemicals needed

- Kodak GBX Developer

Developer 54 ml
↓ ←MQ 190 ml
Mix and fill up to 250 mL
↓
Keep it at 17ºC

*Dilute it every time. Color of diluted solution should be from clear to very light yellow. If the original reagent becomes brownish, do not use it and order new.

Ilford Fixer (250 ml)

Chemicals needed

- ILFORD HYPAM

Fixer 50 ml
↓ ←MQ 200 ml
Mix and keep it at 17ºC

Ilford Developer (250 ml)

Chemicals needed

- ILFORD PHENISOL

Developer 50 ml
↓ ←MQ 200 ml
Mix and keep it at 17ºC

*Same as Kodak, always ensure that the developer is fresh and has not turned brown.

Suggested exposure time for various substrates according to literature

Substrate	Conc. (nM)	Emulsion	Incubation time (h)	Temp. (°C)	Location	Reference
^{3}H-AA mixture (47 Ci/mmol^{-1})	2.1	NTB-2	48	-20	Delaware Bay	Cottrell and Kirchman (2000)
^{3}H-NAG (9,9 Ci/mmol^{-1})	10	NTB-2	48	-20		
^{3}H-Protein [from Vibrio grown on ^{3}H-leucine]		NTB-2	48	-20		
^{3}H-Chitin [from fungus grown on ^{3}H-NAG]		NTB-2	48	-20		
^{3}H-Thymidine (83,5 Ci mmol^{-1})	20	NTB-2	48	4	Delaware estuary	Cottrell and Kirchman (2003)
^{3}H-Leucine (150 Ci mmol^{-1})	20	NTB-2	48	4		
^{3}H-AA mixture (47 Ci mmol^{-1})	0.5	NTB-2	48-144	4	North Atlantic Ocean	Malmstrom et al. (2004a)
^{35}S-DMSP (1,170 Ci mmol^{-1})	<0.1	NTB-2	336-480	4		
^{3}H-Thymidine (83,5 Ci mmol^{-1})	20	NTB-2	28-48	4	Delaware estuary	Cottrell and Kirchman (2004)
^{3}H-Leucine (150 Ci mmol^{-1})	20	NTB-2	3-7	RT		
^{35}S-DMSP (12-43 TBq mmol^{-1})	<0.1	LM-1	48-144	4	North Atlantic Ocean	Malmstrom et al. (2004b)
^{3}H-Protein (0,3-0,9 Ci/gr^{-1}) [from *Vibrio* grown on ^{3}H-Leucine]	20 ng/ml	NTB-2	10 days	4	North Atlantic Ocean	Malmstrom et al. (2005a)
^{3}H-AA mixture (47 Ci/mmol^{-1})	0.5	NTB-2	48-72	4		
^{3}H-Glucose (40 Ci/mmol^{-1})	0.5	NTB-2	48-72	4		
^{3}H-Glucose (33 Ci/mmol^{-1})	2	NTB-2	12-24	4	Delaware estuary	Elifantz et al. (2005)
^{3}H-EPS [from *Nitzschia* grown on ^{3}H-glucose]	1,5	NTB-2	72-144	4		
^{35}S-DMSP (12-43 TBq/mmol^{-1})	<0.1	LM-1	48-144	4	North Atlantic Ocean	Malmstrom et al. (2005b)
^{35}S-MeSH (43 TBq/mmol^{-1})	<0.01	LM-1	48-144	4		

The list is same as on protocol ver. 2007

NAG: N-acetylglucosamine
EPS: extracellular polymeric substances
DMSP: dimethylsulfoniopropionate
MeSH: methanethiol

8. Chemicals –Order information–

Product	Description	Company	Art.Nr.:	Size
Agarose	Low melting point Dnase and Rnase none detected	Sigma-Aldrich	A9414	5 g
Blocking Reagent	For nucleic acid hybridization and detection	Roche Diagnostics GmbH (Sigma-Aldrich)	11 096 176 001	50 g
Buechner funnel	(diam.59mm)	VWR International	HALD127C/1	
Citifluor	Glycerol/ PBS solution AF1	Citifluor Ltd. (Electron microscopy science)	17970	100 ml
DAPI	DAPI stain	Sigma-Aldrich	D9564	1x10 mg
Dextran Sulfate		Sigma-Aldrich	D8906	100 g
EDTA		Plusone	17-1324-01	
Formamide	for molecular biology	Sigma-Aldrich	47671	250 ml
H_2O_2	30% (w/w)	Sigma-Aldrich	H1009	5 ml
Lysozyme	from chicken egg white	Sigma-Aldrich	Nr.: L7651	50000 units/mg, 25 g
maleic acid		Sigma-Aldrich	M-0375	500 g
multiwell plates	12-well (TC treated with lid)	Sigma-Aldrich	M 8687	1St.
N,N-Dimethylformamide (DMF)	N,N-Dimethylformamide	Sigma-Aldrich	227056	100 ml
Nitrocellulose filter	0.45 µm, 25 mm (diameter)	Millipore	HWAP	100 pcs
Polycarbonate filter	0.2 µm, 25 mm (diameter)	Millipore	GTTP	100 pcs
Probes	DNA oligonucleotide probe 5' HRP conjugate Purification: PAGE	Biomers		Scale: S (0.05 µmol)
Proteinase K	(Fluka) frim tritirachium album	Sigma-Aldrich	Nr.: 82456	1x1ml
SDS	Lauryl Sulfate	Sigma-Aldrich	L-4509	100 g
Triethylamine (TEA)	Triethyl amine	Sigma-Aldrich	17924	
Tris(hydroxymethyl)aminomethane (Tris)	Trizma® base	Sigma-Aldrich	T1503	1 kg
Triton X100	X-100	Sigma-Aldrich	9002-93-1	500 ml
Tyramide dye (green)	Alexa Fluor™ 488 NHS Ester (Succinimidyl Ester)	Thermo Fisher Scientific	A20000	1mg
Tyramide dye (orange)	Alexa Fluor™ 555 NHS Ester (Succinimidyl Ester)	Thermo Fisher Scientific	A20009	1 mg
Tyramine hydrochloride (TYR-HCl)		Sigma-Aldrich	T2879	5 g
Vectashield		Vector Laboratories, Inc.	H-1000	10 ml
Water	Sigma Water	Sigma-Aldrich	W4502	1 L

8. Chemicals –Order information–

Chemicals additionally needed for microautoradiography

Product	Description	Company	Art.Nr.:	Size
2 slide mailer	tubes for emulsion	Raymond A Lamb Limited	Nr.:E6.2	125x
Dispensette	0.5-10ml			
Ilford developer	Ilford Phenisol 5L Developer	Ilford (AgarScientific)	CAT1757635, AGP9106 (AgarScientific)	5 l
Ilford emulsion	Nuclear Emulsion Type K5, 0.2µm crystal	AgarScientific	AGP9281	50 ml
Ilford Fixer	Ilford Hypam Fixer	AgarScientific	AGP9183	5 l
Kodak Developer	Carestream® Kodak® autoradiography GBX developer/replenisher	Carestream Kodak (Sigma-Aldrich)	P7042	1GA
Kodak emulsion	Type NTB	Carestream Kodak	889 5666	118 ml
Kodak Fixer	Carestream® Kodak® Processing chemicals (Kodak Fixer)	Carestream Kodak (Sigma-Aldrich)	P6557	1GA
Safetylight	Dunkel kammer leuchte 230V, 50Hz	Kaiser Fototechnik	Nr.: 4018	
Safetylight	lamp model B	Eastman Kodak company	141-2212	
Safetylight filter	Dunkel kammer filter (rot) 9 x12 cm	Hama	Nr.:8194	
Safetylight filter	Kodak 2 darkred	Eastman Kodak company	152-1525	
staining dishes		Sigma-Aldrich	S4642	2x3
staining dishes	slide rack	Sigma-Aldrich	S5017	1x3
staining dishes	rack handle	Sigma-Aldrich	S5142	1x3

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