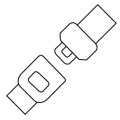
Click-Chemistry

Protocol for Bacteria and Archaea on PC-filters



by Gerhard J. Herndl Lab @ www.microbial-oceanography.eu

 The protocol has been checked, however, if you have suggestions for improvements please let us know (chie.amano@univie.ac.at). Improvement of the protocol is an ongoing process; therefore, this version is not the final version. If needed, FISH or CARD-FISH can be combined. These method can be found in MICRO-CARD-FISH protocol.

1. Incubation and click reaction

Cell sampling, incubation, and fixation

1. Check the abundance of cells in samples to estimate the volume to incubate. For example:

Abundance of cells in sample	Volume to filter (ml)
$1x10^4$ cell ml^{-1}	~100
$5x10^5$ cell ml^{-1}	~10
$1x10^6$ cell ml^{-1}	~5

2. Transfer water samples to Greiner tubes (samples and killed controls). Fix controls with 0.2 µm filtered 37% formaldehyde (final conc. 2-4%) and wait for 15min.
3. Add substrate for both samples and killed controls, e.g.:

Substrate	Final concentration in sample*
HPG	20 nM (open ocean)
EdU	20 nM (open ocean)
AHA	20 nM (open ocean)

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

4. Incubate in the dark at in situ temperature. Take into account the expected level of activity for your sample and the substrate activity (e.g. for bathypelagic with HPG: 20-30h, mesopelagic: 12-20 h, epipelagic: 2-7 h).
5. Kill all samples with 0.2 µm filtered formaldehyde (final conc. 2-4%) and store at 4ºC in the dark for 1 to max 24 h.
6. Filter sample onto white polycarbonate filters, using a cellulose nitrate support filter.
7. After sample filtration, wash the filter twice with 5-10 ml of MQ.
8. Air-dry filters
9. Store at –20ºC until processing. Filters can be stored frozen for several months.

Click-chemistry

*Work under dim light conditions

1. Thaw 10xBuffer additive, and Alexa488 azide at RT in the dark. Keep the 10xBuffer additive on ice.
2. Cut filter sections (1/12 portions of a 25mm diameter filter) and label them with a pencil.
3. Dilute the 10xBuffer additive 1:10 with MQ (e.g., 10 µL of 10xBuffer additive plus 90 µL of MQ), referred to as Buffer additive.
4. Prepare reaction buffer.

Stock reagent	Vol (µl)*	Vol (µl)**
MQ	154	231
10x reaction buffer	20	30
Copper protectant	4	6
Alexa 488 picolyl azide	2	3
Buffer additive	20	30

* total 200 µl in 0.6 ml tube (~10 filter sections)
**total 300 µL in 1.5 ml tube (~15 filter sections)

5. Incubate filter sections in the reaction buffer at RT in the dark for 30 min.
6. After incubation, wash x3 in excess MQ.
7. Place the filter sections on a blotting paper (with sample side up) and dry them at 37 °C in hybridization oven for 10 min. After click reaction, do not expose the sample directly to intense light (you can work in a room with dim illumination, e.g., half of the bulbs on).

FISH or CARD-FISH

*If combining Click-chem with FISH or CARD-FISH, go to our FISH/CARD-FISH protocol. Start from embedding step. Note that Embedding shouldn't be done before click reaction, otherwise you'll get very high background noise.

Internal standard

*Optionally you can add the internal standard.

1. Sonicate beads solution (0.3% intensity, 1:10 dilution with Sigma water) for 10 min.
2. Immediately after sonication, pipette 5 µl drops (one drop for each filter section) of the beads solution onto clean cover slips (10 drops/ cover slip) and place the filter sections on top of the beads´ drop (with the sample side down).
3. Let the filters dry at 37 ºC in hybrid. oven for 15 min in the dark.
4. After drying filters, carefully remove filters from the cover slip.

Cell transfer

* Since the reflection of the beam of the fluorescence microscope on white polycarbonate filter disturb the detection of weak signals and we prefer to have dark background, our procedure includes the cell transfer step especially for the deep sea samples. This step is optional, depending on your samples.

1. Prepare gelatin solution in 50 ml Greiner tube.

Stock reagent	Volume	Final
Gelatin	0.6 g	3%
MQ	20 mL	

2. Warm up the gelatin solution at 43°C for 15min until it dissolves
3. Dip a slide glass into the gelatin solution to coat
4. Wipe off the gelatin only from the back side of the slide. Place the gelatin-coated slide on an ice-cold aluminium plate for 1-5 min to solidify. The time depends on the laboratory's humidity. Check carefully to make sure it doesn't dry out.
5. Place the sample filter sections on the gelatin-coated slide (sample side down, max ~12 filter pieces on a slide). Add a new filter piece as a control for gelatin.
6. Dry the slide at RT for 20min-1h in the dark, until the gelatin is completely dry.
7. Mark the filter location on the side without the sample filter using a permanent marker. Write down the sample ID, as well.
8. Carefully wet the edge of filter with a MQ-wetted cotton swab, if needed. Hold the wet edge carefully with the forceps and gently (very slowly) peel off the filter from the slide.
9. Mount with DAPI-mix and keep at -20 ºC until taking photos with a camera attached to a microscope.

Ref

Click-chemistry: Samo et al. (2014), Hatzenpichler et al (2014), Hatzenpichler and Orphan (2015)

2. Buffers and Chemicals

10x Buffer additive

1. Prepare sterile 0.6 mL eppis (e.g. by UV)

Stock reagent	Volume	Final
Buffer additive (Component E)	powder	10x
Sigma water	2 ml	

2. Add the water into the bottle and mix until completely dissolved.
3. Make 100-200 µL aliquots
4. Store the stock solution at ≤–20°C. This stock solution is stable for up to 1 year.

Alexa Fluor picolyl azide (PCA) stock (1000 µM)

Stock reagent	Volume	Final
Alexa 488 PCA (Component A)	1 tube	1000 µM
DMSO	105 µL	

Make aliquots in black eppis and store at -20 °C

CuSO4 copper protectant pre-mix

Stock reagent	Volume	Final
CuSO4 (Component C)	200 µL	2:1
Copper protectant (Component D)	100 µL	

DAPI 50 µg/ml – 500 µl:

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

*if DAPI is difficult to dissolve in PBS, it can be dissolved in Sigma water

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

10x PBS - 500 ml:

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

Web pages:

- Click-iT™ Plus Alexa Fluor™ 488 Picolyl Azide Toolkit, C10641 ThermoFIsher Scientific, 604EUR/ 1kit https://www.thermofisher.com/order/catalog/product/C10641?SID=srch-srp-C10641

*The prices are in 2025. They may be changed

3. Product information

Ordering info

Product	Description	Company	Art.Nr.:	Size
Alexa picolyl Azide	Click-iT™ Plus Alexa Fluor™ 488 Picolyl Azide Toolkit	Thermo FIsher Scientific	C10641	1 kit
Buechner funnel	(diam.59mm)	VWR International	HALD127C/1	
Citifluor	Glycerol/ PBS solution AF1	Citifluor Ltd. (Electron microscopy science)	17970	100 ml
Click-iT® Protein Synthesis Assay Kit*	Click-iT® HPG Alexa Fluor® 488 Protein Synthesis Assay Kit	Thermo FIsher Scientific	C10428	
DAPI	DAPI stain	Sigma-Aldrich	D9564	1x10 mg
DMSO	Dimethyl sulfoxide for molecular biology (DMSO)	Sigma-Aldrich	D8418-50ML	50 mL
Gelatin	Gelatin from bovine skin Type B, powder, BioRegent, suitable for cell culture	Sigma-Aldrich	G9391	100G
Polycarbonate filter	0.2 µm, 25 mm (diameter)	Millipore	GTTP	100 pcs
Vectashield		Vector Laboratories, Inc.	H-1000	10 ml
Water	Sigma Water	Sigma-Aldrich	W4502	1 L

* we only need HPG from this kit, when we use Click-iT™ Plus Alexa Fluor™ 488 Picolyl Azide Toolkit

General Lab equipment, materials

- Water bath
- Forceps
- Slide glass
- Cover slip (18x18mm, 24x60mm)
- Blade
- Pencil
- Permanent marker
- Petri dish
- Blotting paper, tissue paper
- Fluorescence microscope (x1000 magnification)
- Microscopy immersion oil
- 70% Ethanol for cleaning
- MQ
- Greiner tubes (50 mL)
- Eppis (0.6 ml, 1.5 ml)