Oct 18, 2022 Version 1

SHInE - Simultaneous HCR, Immunohistochemistry, Nuclear staining and EdU V.1

Integrative and Comparative Biology

DOI
dx.doi.org/10.17504/protocols.io.5qpvobnyzl4o/v1

Aida Coric[1,2], Alexander W. Stockinger[1,2,3], Petra Schaffer[1,2], Dunja Rokvic[1,2], Kristin Tessmar-Raible[1,2,4,5], Florian Raible[1,2,3]

[1]Max Perutz Labs, University of Vienna, Vienna BioCenter, Dr. Bohr-Gasse 9/4, 1030 Vienna, Austria;
[2]Research Platform "Rhythms of Life", University of Vienna, Vienna BioCenter, Dr. Bohr-Gasse 9/4, A-1030 Vienna, Austria;
[3]Research Platform "Single-Cell Genomics of Stem Cells", University of Vienna, Vienna BioCenter, Dr. Bohr-Gasse 9/4, A-1030 Vienna, Austria;
[4]Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany;
[5]Carl-von-Ossietzky University, Carl-von-Ossietzky-Straße 9-11 ,26111 Oldenburg, Germany

Alexander Stockinger
University of Vienna

DOI: https://dx.doi.org/10.17504/protocols.io.5qpvobnyzl4o/v1

Protocol Citation: Aida Coric, Alexander W. Stockinger, Petra Schaffer, Dunja Rokvic, Kristin Tessmar-Raible, Florian Raible 2022. SHInE - Simultaneous HCR, Immunohistochemistry, Nuclear staining and EdU. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.5qpvobnyzl4o/v1**Version created by **Alexander Stockinger**

Manuscript citation:
Aida Ćorić, Alexander W Stockinger, Petra Schaffer, Dunja Roković, Kristin Tessmar-Raible, Florian Raible, A Fast And Versatile Method for Simultaneous HCR, Immunohistochemistry And Edu Labeling (SHInE),*Integrative and Comparative Biology*, 2023;, icad007,https://doi.org/10.1093/icb/icad007

Protocol status: Working
We use this protocol and it's working

Created: October 10, 2022

Last Modified: October 18, 2022

Protocol Integer ID: 71099

Keywords: HCR, immunohistochemistry, in situ hybridisation, FISH, whole-mount, multiplexed, EdU, proliferation, Platynereis, Annelid, posterior regenerates of the bristleworm platynereis dumerilii, bristleworm platynereis dumerilii, mount platynereis tissue, different molecular labelling technique, multiplexed zoo of mrna expression, detection of dna, mount tissue sample, nuclear staining, hoechst staining, sensitive detection of dna synthesis, gene expression, various developmental stages of the animal, germline cluster proliferation, molecules in the same specimen, posterior regenerate, simultaneous hcr, detected molecule

Abstract

This protocol allows the multiplexed use of four different molecular labelling techniques in whole-mount *Platynereis* tissues. In short, gene expression (via in situ HCR 3.0), cell proliferation (via EdU labelling), proteins (via Immunohistochemistry) and nuclei (DAPI/Hoechst staining) can be assessed in whole-mount tissue samples, allowing the co-visualisation of various types of molecules in the same specimen.

This protocol combines knowledge from multiple sources (see below), and is being submitted in parallel with a manuscript detailing the applications of the method. (We will add the reference as soon as it's available.)

We successfully applied this protocol to heads and posterior regenerates of the bristleworm *Platynereis dumerilii*, at various developmental stages of the animal. Given the general nature of the detected molecules, and the wide-spread use of the individual detection techniques, we anticipate that this protocol will be well applicable to a wider range of model systems.

References:
1) Choi HMT, Calvert CR, Husain N, Huss D, Barsi JC, Deverman BE, Hunter RC, Kato M, Lee SM, Abelin ACT, Rosenthal AZ, Akbari OS, Li Y, Hay BA, Sternberg PW, Patterson PH, Davidson EH, Mazmanian SK, Prober DA, Rijn M van de, Leadbetter JR, Newman DK, Readhead C, Bronner ME, Wold B, Lansford R, Sauka-Spengler T, Fraser SE, Pierce NA. 2016. Mapping a multiplexed zoo of mRNA expression. *Development* 143:3632–3637. doi:10.1242/dev.140137

2) Choi HMT, Schwarzkopf M, Fornace ME, Acharya A, Artavanis G, Stegmaier J, Cunha A, Pierce NA. 2018. Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development* 145:dev165753. doi:10.1242/dev.165753

3) Kuehn E, Clausen DS, Null RW, Metzger BM, Willis AD, Özpolat BD. 2021. Segment number threshold determines juvenile onset of germline cluster proliferation in Platynereis dumerilii. *Biorxiv* 2021.04.22.439825. doi:10.1101/2021.04.22.439825

4) Pende M, Vadiwala K, Schmidbaur H, Stockinger AW, Murawala P, Saghafi S, Dekens MPS, Becker K, Revilla-i-Domingo R, Papadopoulos S-C, Zurl M, Pasierbek P, Simakov O, Tanaka EM, Raible F, Dodt H-U. 2020. A versatile depigmentation, clearing, and labeling method for exploring nervous system diversity. *Sci Adv* 6:eaba0365. doi:10.1126/sciadv.aba0365

5) Salic A, Mitchison TJ. 2008. A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc National Acad Sci* 105:2415–2420. doi:10.1073/pnas.0712168105

6) Tessmar-Raible K, Steinmetz PRH, Snyman H, Hassel M, Arendt D. 2005. Fluorescent two-color whole mount in situ hybridization in Platynereis dumerilii (Polychaeta, Annelida), an emerging marine molecular model for evolution and development. *Biotechniques* 39:460–464. doi:10.2144/000112023

Guidelines

- Please use aliquots of common stocks, including all buffers, to avoid repeated freeze-thaw cycles.
- The protocol is based on the detection of RNA, so minimize RNA degradation. It does not seem to be as critical as with sequencing experiments, but keep samples on ice whenever indicated, and work quickly in a clean setting.
- Protect samples from light once fluorescent dyes have been added to them!
- HCR signal seems to be best in the first 5 days after preparation of the samples, so ideally, image your samples right after preparing them.
- Many of the steps in this protocol can be optimized for individual samples or experimental conditions. We tried to indicate these steps. If you do come up with improved conditions for certain samples, we want to encourage you to upload a modified version of this protocol!

For multi-color fluorescence labeling, check whether the microscope you intend to use has lasers and filters available. HCR signal in general seems to only be visible on the confocal microscope. You can also check for bleed through risk and plan accordingly, for example using this tool:
https://www.thermofisher.com/order/fluorescence-spectraviewer

Probe maker:
Probes can be ordered from Molecular Instruments, alongside the amplifier hairpins that contain the fluorophore. To design HCR probes yourself, you can use a Python based tool developed by Ryan Null in the Özpolat lab at WUSTL. It can be found here:
https://github.com/rwnull/insitu_probe_generator
and was first referenced in
Kuehn E, Clausen DS, Null RW, Metzger BM, Willis AD, Özpolat BD. 2022. Segment number threshold determines juvenile onset of germline cluster expansion in Platynereis dumerilii.*J Exp Zoology Part B Mol Dev Evol*338:225–240. doi:10.1002/jez.b.23100

In short, use a target sequence and the probe generator will generate sets of probes that contain the initiator sequence of your choice.

* Probes can either be acquired through https://www.molecularinstruments.com, or self-designed (we use this python tool https://github.com/rwnull/insitu_probe_generator) and then ordered as oligos, for example through https://www.idtdna.com/pages/products/custom-dna-rna/dna-oligos/custom-dna-oligos/opools-oligo-pools

For more detailed instructions on EdU pulse-and-chase experiments in *Platynereis dumerilii*, please refer to Zattara, E. E. & Özpolat, B. D. Developmental Biology of the Sea Urchin and Other Marine Invertebrates.*Methods Mol Biology***2219**, 163–180 (2020).

Materials

Buffer recipes

Hybridization buffer (toxic, store at -20° C):

	1x concentration	For 40ml
	30% formamide	12 mL formamide
	5x sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
	9 mM citric acid (pH 6.0)	360 µL 1 M citric acid, pH 6.0
	0.1% Tween 20	400 µL of 10% Tween 20
	50 µg/mL heparin	200 µL of 10 mg/mL heparin
	1x Denhardt's solution	800 µL of 50× Denhardt's solution
	10% dextran sulfate	8 mL of 50% dextran sulfate
		Fill to 40 ml with ultrapure H2O

Wash buffer (toxic, store at -20° C):

	1x concentration	For 40ml
	30% formamide	12 mL formamide
	5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
	9 mM citric acid (pH 6.0)	360 µL 1 M citric acid, pH 6.0
	0.1% Tween 20	400 µL of 10% Tween 20
	50 µg/mL heparin	200 µL of 10 mg/mL heparin
		Fill up to 40 ml with ultrapure H2O

Amplification buffer (store at 4° C):

	1x concentration	For 40ml

5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 µl of 10% Tween 20
10% dextran sulfate	8 mL of 50% dextran sulfate
	Fill up to 40 ml with ultrapure H2O

5x SSCT (store at 4° C):

1x concentration	**For 40ml**
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0,1% Tween 20	400µl of 10% Tween 20
	Fill up to 40µl with ultrapure H2O

1X PTW:
1X PBS with 0.1% Tween-20

Tissue Clearing:
1.Pende, M.*et al.* A versatile depigmentation, clearing, and labeling method for exploring nervous system diversity.*Sci Adv***6**, eaba0365 (2020).

Reagents

A	B	C
Reagent	**Manufacturer**	**Product Number**
Citric acid monohydrate	Sigma-Aldrich	C1909
Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 488 dye	Invitrogen™	C10337
Denhardt's solution 50x	Invitrogen™	750018

	A	B	C
	Dextran Sulfate 50% solution	Merck Millipore	S4030
	Formamide	Sigma-Aldrich	47671
	Glycine	Roth	3908.3
	Heparin	Sigma-Aldrich	H3393
	Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water	Invitrogen™	H3570
	Magnesiumchlorid hexahydrate ($MgCl_2$)	VWR	25.108.295
	Methanol	VWR	20847
	Methanol, suitable for HPLC	Sigma-Aldrich	34860
	Paraformaldehyde (PFA)	Sigma-Aldrich	441244
	Phosphate buffered saline (PBS)	Sigma-Aldrich	P4417
	Proteinase K	Sigma-Aldrich	1.245.680.100
	Saline-Sodium Citrate buffer 20× Concentrate (SSC 20X)	Sigma-Aldrich	S6639
	SlowFade™ Diamond Antifade Mountant	Molecular Probes™	S36972
	Thymidine	Sigma-Aldrich	T9250
	Tween 20	Sigma-Aldrich	P1379

Troubleshooting

Optional: EdU incubation

1 Incubate worms with [M] 10 micromolar (µM) EdU in artificial sea water (ASW) (EdU aliquots in both DMSO and H2O work fine) for your desired pulse length.

2 Optional: Chase out EdU with [M] 3 millimolar (mM) thymidine for 01:00:00 ; then replace thymidine solution with fresh ASW and incubate for desired chase length

1h

Day 1: Fixation, Dehydration

3 **Dissect tissue of interest**
Anesthetize worms in a 50% mix of 7.5% (w/v) MgCl2 and ASW
Dissect the tissue of interest; transfer it to a 1,5ml microcentrifuge tube pre-filled with 1ml ASW On ice

Note: the protocol is also compatible with whole animals, but will require larger volumes and more reagent accordingly.

4 **Fix the samples**
Replace the ASW in the tube with 1ml 4%PFA/1XPTW (carefully pipet away the ASW; samples should sink to the tube bottom). Fix samples for 01:00:00 at Room temperature shaking gently on a rocking platform

1h

Make sure the samples are swimming back and forth freely in the tube; dislodge stuck samples by gently flicking the tube.

5 **Dehydrate and store samples**
Dehydrate the samples through a series of increasing MeOH concentrations diluted in 1X PTW.
Perform these steps on ice, and wait until the samples have settled down at the bottom of the well after each increase in MeOH concentration (takes around 1-3 minutes)

11m

- 25% MeOH in PTW 00:03:00
- 50% MeOH in PTW 00:03:00
- 75% MeOH in PTW 00:03:00
- Wash in100% MeOH 00:01:00
- Wash in100% MeOH 00:01:00

6 Store samples at -20° C for at least Overnight . Longer storage is possible (several months in our hands, but even longer storage might be possible).

Day 2: Rehydration, digestion, probe hybridization

1h 45m

7 **Re-hydrate samples** through a series of decreasing MeOH concentrations in 1X PTW on ice. 12m

- 75% MeOH in PTW 00:03:00
- 50% MeOH in PTW 00:03:00
- 25% MeOH in PTW 00:03:00
- Wash 2x in 1X PTW 00:03:00

8 **Perform proteinase K treatment** with 1ml of Proteinase K in PTW according to table below, at Room temperature .

8.1 This is a **critical, time-sensitive step** and all reagents needed for washes after digestion should be prepared in advance. Concentrations and digestion times **should be adjusted to individual tissues** and possibly differences in proteinase K batches.

	Tissue	PK concentration	time
	Heads, Blastemas, adult tissues	100µg/mL	5'
	Larvae, <1dpf	100µg/mL	30 seconds
	Larvae, 3dpf	100µg/mL	2'

8.2 Briefly rinse 2x with 1 ml of glycine wash buffer to stop digest. Work On ice from here on. 5m
Wash once for 00:05:00 with 1x PTW.

8.3 Post-fix in 1mL 4%PFA diluted in 1xPTW for 00:20:00 shaking while still On ice . 20m

8.4 Wash samples 2x 00:05:00 in 1ml PTW. 5m

9 **Optional: tissue clearing** 20m
If clearing with DEEP-CLEAR is wanted, perform it at this step. Worm tissue can be cleared at 37 °C for 00:15:00 at 300 rpm on a heat block. Clearing time should be optimised for different tissues. Wash the samples in PTW thoroughly after clearing (3x 00:05:00).

Note: in Platynereis tissues, the effects of clearing depend on the tissue sampled. Opaque tissues such as eyes benefit from clearing, while naturally transparent tissues like blastemas don't.

For details on tissue clearing, please refer to
Pende, M.*et al.* A versatile depigmentation, clearing, and labeling method for exploring nervous system diversity.*Sci Adv***6**, eaba0365 (2020)

10 **Probe hybridization** with HCR probe(s) of choice.

Note: a weak probe signal might be improved by increasing the probe concentration; several labs have reported successfully reusing the probe solution (simply freeze the probe mix after use)

10.1 Incubate in 1ml 50% hybridization buffer in PTW for 00:05:00 at 1h 5m
Room temperature
Incubate in 300µl 100% hybridization buffer for 01:00:00 at 37 °C on a heat block .

Careful when replacing media to not lose any samples. The buffer is very viscous; wait until samples settle down to the bottom of the tube.

10.2 Meanwhile prepare the probe solution:

- 1pmol of each probe mix (1µl of 1µM stock) in 250µl hybridization buffer
- heat the probe solution to 37°C (for small volumes, we use the heat block the samples are already on; for larger volumes, we use a water bath)

10.3 Replace the hybridization buffer with the prepared probe solution and incubate the samples Overnight at 37 °C .

11 **Wash out the HCR probes**: 1h 10m

Wash for 00:15:00 with 1ml pre-heated probe wash buffer at 37 °C

Wash for 00:15:00 with 1ml pre-heated probe wash buffer at 37 °C

Wash for 00:15:00 with 1ml pre-heated probe wash buffer at 37 °C

Wash for 00:15:00 with 1ml pre-heated probe wash buffer at 37 °C

Wash for 00:05:00 with 1ml 5X SSCT at Room temperature

Wash for 00:05:00 with 1ml 5X SSCT at Room temperature

From here on, the protocol progresses differently for each combination of methods (HCR only, HCR & EdU, HCR & IHC, HCR, EdU & IHC)
Please select the step case below accordingly:

STEP CASE

HCR only 6 steps

This is the bare-bones protocol to just stain your samples with HCR.

Day 3: HCR probe detection 1h 16m 30s

12 **HCR Amplification:**

Day 3: HCR probe detection 1h 16m 30s

12.1 Wash with 1ml PTW for 00:05:00 at Room temperature 35m

Equilibrate samples in 300µl amplification buffer for 00:30:00 at Room temperature

12.2 **Meanwhile**: 31m 30s

Prepare the HCR hairpin mix.

- Use 15 pmol per hairpin (5µl of 3µM stock) - move them to individual 1,5ml microcentrifuge tubes*
- Heat the hairpins to 95 °C for 00:01:30 by adding them to a pre-heated heatblock
- Let the hairpins cool to Room temperature protected from light for 00:30:00
- Mix the cooled hairpins with amplification buffer to a total volume of 250µl

** NOTE: for each amplifier, TWO different hairpins have to be used! (e.g., B1H1 and B1H2 are a pair of hairpins compatible with the B1 amplifier sequence) - heat those in separate tubes and pool only directly before adding them to the sample!*

** NOTE: several probe pairs can be co-detected in one sample by using different sets of amplifiers with different fluorophores.*

12.3 Incubate the samples in 250 µl hairpin mix Overnight protected from light at Room temperature .

Note: amplification buffer is viscous; samples will eventually equilibrate and sink to the bottom of the tube, but careful pipetting is recommended to not lose any samples.

Day 4: Amplification Termination 1h 25m

13 **Terminate the amplification:** 1h 40m

Wash in 1ml 5X SSCT for 00:05:00 at Room temperature

Wash in 1ml 5X SSCT for 00:05:00 at Room temperature

Wash in 1ml 5X SSCT for 01:00:00 at Room temperature

Wash in 1ml 5X SSCT for 00:05:00 at Room temperature

Wash in 1ml PTW for 00:05:00 at Room temperature shaking on rocking platform

Staining of nuclei (DAPI/ Hoechst) can be performed here by adding the dye directly to PTW and increasing the incubation time to 00:15:00 .

Wash in 1ml PTW for 00:05:00 at Room temperature shaking on rocking platform

14 **Mount the samples:**
Mounting strategies may differ based on sample type/ size.
For whole mount *Platynereis* blastemas or heads, the following works well:

Equilibrate the sample in a small volume of mounting medium (in our hands, SlowFade Diamond worked best)
Put four small pieces of "pattafix" or similar poster mounting clay (Blu-tack or similar) into the corners of the "mounting area" of a microscope slide to build a stage.
Put the sample in the middle of the stage, and add a small volume (~10µl) of mounting medium on top of it.

Gently press down a cover glass, using a second microscope slide to keep it even.