Semi-quantitative analysis of cell proliferation in Asterias rubens *cell monolayers*

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Cell proliferation in sea stars can be investigated both in tissue sections and in monolayers of freshly dissociated cells by using the substitute nucleotide 5-Bromo-2´-deoxyuridine (BrdU) as a mitotic tracer. The following protocol for cell monolayers from a variety of sea star tissues was approved.

1. *A. rubens* were injected with 150 µl of BrdU (10 µg/ml). After 4 h of treatment (or more if wished), the sea stars were removed from the water and dissected.
2. Coelomic fluid was collected in Falcon tubes and the axial organ, coelomic epithelium and Tiedemanns´s body were dissected and transferred to eppendorph tubes with 600 µl of 0.1% collagenase type I and 0.1% collagenase type IV dissolved in CMFSS buffer. After approximately 1 h at RT, released cells were collected by centrifugation (5 min, 2500*g*, RT) and remaining tissues were removed by washing the pellet twice in 500 µl CMFSS buffer.
3. 180 µl of cell suspension was added to 20 µl of 200 mM $CaCl_2$ on SuperFrostPlus slides and left for 45 min at RT to attach to the glass. The attached cells were fixed by submerging the slides into ice-cold 70% ethanol in 50 mM glycine for 5 min.
4. Permeabilisation of the cells was done by incubation in 2 M HCl with 0.2 mg pepsin per ml for 30 min at 30ºC and then neutralised in 0.1 M Na-borate (pH 8.5) for 5 min at RT. Bovine serum albumin (0.5 %) in PBS-tween (0.05% Tween-20) was used as blocking medium, 1 h in a humid chamber at RT before incubation for 30 min with ant-BrdU-FITC (BD Bioscience, 347583) diluted 1:10 in blocking medium. To enhance the FITC labelling the slides were incubated in the same way with a secondary anti-mouse IgG-FITC (Sigma F3008), diluted 1:100 in blocking medium, before washing and mounted in Vectashield with propidiumiodide (H-1300; Vector). Each step of the procedure was followed by rinsing the slides several times in PBS-tween to reduce background staining that otherwise interfere with scoring.
5. Slides were manually examined using a Leica DMRBE fluorescent microscope. The number of BrdU incorporated cells (green cells) was estimated in relation to the number of total cells (red cells) to give a % value. Approximately 300 cells were scored on each slide.

Statistical consideration: Comparisons of % BrdU incorporated nuclei found on cell monolayers from different organs shall ideally be statistically analyzed with a non-parametric test such as Mann–Whitney Rank Sum Test.

Sea stars: Specimens of A. rubens are collected by snorkelling or scuba divers and maintained in basins with running seawater at 15°C and 33 PSU salinity.

Microscope: Leica DMRBE fluorescence microscope equipped with 20, 40 and 100x magnification, filters for visualising propidiumiodide (N2.I-green) and FITC (I3-blue).

Solutions: CMFSS buffer (435 mM NaCl, 10.7 mM KCl, 27 mM Na_2SO_4, 16.6 mM $C_6H_{12}O_6$, 12 mM HEPES pH 7.4); Phosphate-buffered saline solution (PBS: 137 mM NaCl, 2.7 mM KCl, 15.8 mM Na_2HPO_4, 1.23 mM KH_2PO_4; pH 7.4).

Labware: 15 ml Falcon tubes and Eppendorf tubes (all sterile). Scissors and forceps, SuperFrost®Plus slides and cover slips.

Additional information:

***Söderhäll I, Bangyeekhun E, Mayo S, Söderhäll K** (2003) Hemocyte production and maturation in an invertebrate animal; proliferation and gene expression in hematopoietic stem cells of Pacifastacus leniusculus. Dev Comp Immunol 27:661-672*

***Holm K, Dupont S, Nilsson Sköld H, Stenius A, Thorndyke M, Hernroth B** (2008) Induced cell proliferation in putative haematopoietic tissues of the sea star, Asterias rubens (Linnaeus). J Exp Biol. 211:2551-2558*

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