Chapter 20

Studying Stem Cell Biology in Intact and Whole-Body Regenerating *Hydra* by Flow Cytometry

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Abstract

The freshwater *Hydra* polyp is a versatile model to study whole-body regeneration from a developmental as well as a cellular point of view. The outstanding regenerative capacities of *Hydra* are based on its three populations of adult stem cells located in the central body column of the animal. There, these three populations, gastrodermal epithelial, epidermal epithelial, and interstitial, continuously cycle in homeostatic conditions, and their activity is locally regulated after mid-gastric bisection. Moreover, they present an unusual cycling behavior with a short G1 phase and a pausing in G2. This particular cell cycle has been studied for a long time with classical microscopic methods. We describe here two flow cytometry methods that provide accurate and reproducible quantitative data to monitor cell cycle regulation in homeostatic and regenerative contexts. We also present a cell sorting procedure based on flow cytometry, whereby stem cells expressing a fluorescent reporter protein in transgenic lines can be enriched for use in applications such as transcriptomic, proteomic, or cell cycle analysis.

Key words *Hydra*, Cell cycle, Adult stem cells, Epithelial stem cells, Interstitial stem cells, Flow cytometry, GFP cell sorting, Click-iT EdU labeling of regenerating animals, Cell cycle analysis

1 Introduction

1.1 Hydra and the Unusual Properties of Its Stem Cells

The hydrozoan *Hydra* polyp, which belongs to Cnidaria, is well known for its robust regenerative abilities, thus providing an attractive model system for regenerative biology [1–5]. This freshwater polyp, which is about 1-cm long, reconstructs any missing part of its body, such as the basal (foot) or apical (head) regions, within a few days after amputation. The *Hydra* body exhibits a radial symmetry (Fig. 1a), with a cylindrical shape terminated at the apical pole by the head region centered on a unique opening called mouth, and at the basal pole by the basal disc, which helps the animal to adhere to various environmental substrates. The anatomy of *Hydra* is formed of two tissue layers, one epidermal, the other gastrodermal, held together by the mesoglea, a complex extracellular matrix that maintains their cohesion. This double-body wall

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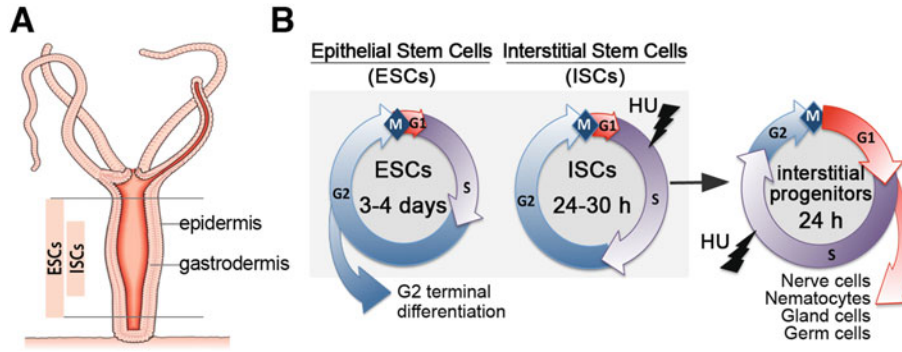

Fig. 1 *Hydra* anatomy and cycling properties of *Hydra* stem cells. (**a**) The *Hydra* polyp exhibits a radial symmetry centered on an oral–aboral axis. At the apical pole also called the head, a ring of tentacles surrounds a dome called hypostome that is centered on the mouth opening, while at the basal end called foot, the basal disc that produces mucus allows the animal to attach to substrates. The animal consists of two epithelial layers, the epidermis on the outside consisting of epidermal epithelial stem cells (eESCs), and the gastrodermis on the inside consisting of gastrodermal epithelial stem cells (gESCs). All epithelial cells along the gastric region are ESCs that terminally differentiate when they reach the extremities. Interstitial stem cells (ISCs) are abundant in the central region of the animal, interspersed with eESCs. (**b**) The cell cycle of ESCs lasts 3–4 days, while multipotent ISC cycle every 24–30 h. In each cycle, ISCs provide asymmetrically divided interstitial progenitors (IPs) that cycle faster than ISCs (less than 24 h). IPs are migratory cells that differentiate in G0 phase along the body axis and at the extremities. As for somatic derivatives, nematocytes are strictly located in the epidermis, nerve cells are found in both layers, and gland cells in the gastrodermis. Fast cycling cells such as ISCs and IPs are predominantly killed by hydroxyurea (HU) pulse treatment. In contrast, ESCs paused in G2 are resistant to such treatments. Passively moving toward the apical and basal poles, ESCs differentiate in G2 phase into head- and foot-specific epithelial cells. *Scheme adapted after* [6]

houses three populations of non-interchangeable adult stem cells: two populations of unipotent epithelial stem cells (ESCs), either epidermal or gastrodermal, and the multipotent interstitial stem cells (ISCs), which are found predominantly in the central body column [7–10] (Fig. 1a). By contrast, the tissue at both the ends of the animal consists mainly of differentiated cells, either epithelial or derived from ISCs, such as neurons and mechano-sensory cells called nematocytes [11, 12]. In addition, ISCs can also differentiate into glandular cells, distributed among the gastrodermal epithelial cells, and germ cells when the animal becomes sexual and differentiates gonads [9, 13].

Hydra tissue is characterized by a dynamic homeostasis, as stem cells from the central body region from all three lineages are continuously self-renewing, replacing every 20 days the differentiated cells that progressively get sloughed off at the extremities [14]. The length of the cell cycle of these adult stem cells differs between ESCs that divide every 3–4 days and ISCs that progress faster through the cell cycle, dividing every 24–30 h (Fig. 1b) [15, 16]. Intriguingly, all three stem cell populations share quite unusual features, i.e., a very short G1 phase that lasts 1 h, and an extended G2 phase that ranges from 24 up to 72 h for ESCs [6, 15,

17] and from 6 up to 22 h for ISCs [15, 16]. As a consequence, pulse treatments with drugs that block DNA synthesis such as hydroxyurea (HU) preferentially target the fast cycling cells, the ISCs, and their progenitors (Fig. 1b). Typically, three 24-h periods of HU exposure, each separated by a 12-h drug-free period, suffice to selectively deplete the interstitial cell line and, after a few weeks, produce animals that are purely epithelial. These animals, which have lost their nervous system, cannot catch preys yet can survive for months and years if manually force-fed. In such conditions, the epithelial tissues get properly renewed and the animals retain their developmental potential, i.e., are able to bud and regenerate [18, 19].

The continuous self-renewal of *Hydra* stem cells in the central part of the body column indicates that this region can be considered as a *pro-blastema* where stem cells paused in G2-phase are ready to differentiate, divide, and proliferate immediately after amputation [20, 21]. Similar to other cnidarians [22–24], proliferating cells play an important role in the regeneration of apical structures, and a synchronous cell division event is actually rapidly induced upon amputation [20, 25]. Seventeen evolutionarily conserved cell cycle genes are then synchronously upregulated (*Shox1, E2F7, TFDP1, POLQ, CCNF, PLK4, CCNE1, CCND2, CDC7, SIPA1L3, MCM5, DLEC1, MCM9, CDC6, CCNA2, CCNB3, PLK1*) [20], and a local wave of cell proliferation follows at an early-late stage (around 24 h post-amputation) [26]. When the S-phase progression is blocked with HU prior to amputation, it alters apical regeneration, although only partially, as the stock of epithelial cells stopped in G2 can differentiate without entering a final mitosis [21]. In summary, the regenerative capacity of *Hydra* relies on large stocks of continuously cycling stem cells, whose unusual properties explain their immediate contribution to the regenerative process, which is achieved in few days.

1.2 Stem Cell Sorting and Methods to Monitor the Cell Cycle Activity in Hydra

The cell cycling behavior of *Hydra* stem cells was intensively studied by classical microscopical methods that either analyze the incorporation of thymidine analogs (e.g., ^{3}H-thymidine, BrdU 5′-bromo-deoxyuridine) into replicating DNA, or evaluate the DNA content microfluorimetrically, or allow the counting of mitotic figures [16, 17, 27]. Among these methods, the counting of BrdU-labeled nuclei detected with anti-BrdU antibody allows the establishment of a precise BrdU-labeling index [26, 28]. However, these methods are time-consuming, and the quantitative results are obtained on a rather small number of analyzed cells. To overcome these limitations, we have applied to *Hydra* flow cytometric methods that are commonly used in model organisms such as algae, sea anemones, planarians, flies, as well as in mammalian cells, to address a variety of biological questions [29–33].

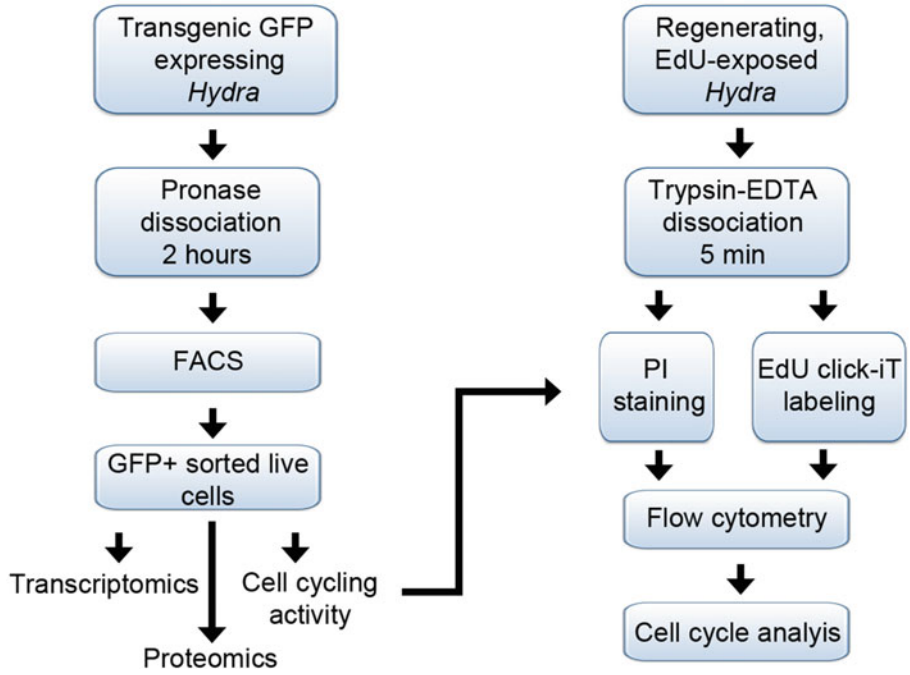

Fig. 2 Schematic view of the flow cytometry protocols presented in this chapter. In this study, two distinct protocols were used to dissociate the *Hydra* tissues, the first one is based on pronase activity and the second on trypsin–EDTA activity. Pronase dissociation is suitable because it provides living, viable *Hydra* cells that can be sorted by flow cytometry (*see* Subheading 3.2). The trypsin–EDTA dissociation is fast and convenient to use when rapid dissociation is needed as required to analyze the cell cycle activity (*see* Subheadings 3.3 and 3.4)

As a general definition, flow cytometry is a laser-based technology that allows the characterization of properties of cells in suspension, i.e., their size, volume, morphological complexity, and fluorescence-labeled components. This technology ensures that a large number of cells are analyzed quantitatively in a very short time. Advances of the flow cytometry-based cell sorting methodology (FACS, fluorescent-activated cell sorting) opened large possibilities for downstream applications such as transcriptomic [34, 35], proteomic [36], biochemical, or cellular [33] analyses. In this context, the production of transgenic lines in *Hydra* that constitutively express green fluorescent protein (GFP) in one or the other stem cell population, e.g., *ecto-GFP* [37], *endo-GFP* [38], or *Cnnos1*-GFP [39], opened the possibility to study the molecular signatures of each population after cell sorting [39–41]. The two first sections in this chapter describe how to sort *Hydra* stem cells based on their selective GFP expression (Figs. 2 and 3).

The flow cytometry methodology is also applied in *Hydra* to measure the modulations of the cell cycle behavior in homeostatic, regenerative, or ecotoxicological contexts [6, 20, 42–44]. A simple and easy method to characterize the distribution of cell populations in the different phases of the cell cycle is to measure the DNA

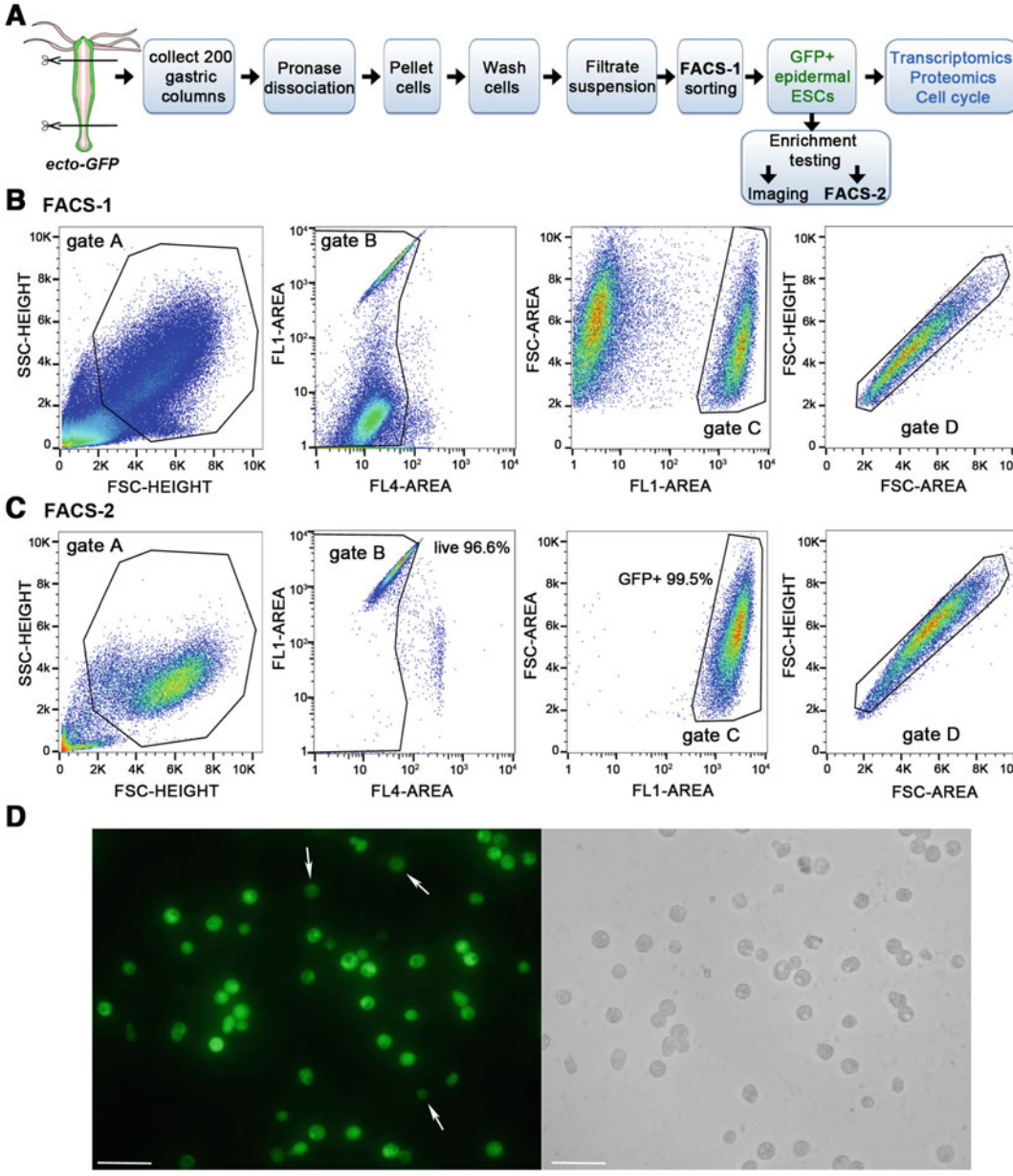

Fig. 3 Fluorescence-activated cell sorting (FACS) of epidermal ESCs constitutively expressing GFP. (**a**) Scheme depicting the FACS procedure to obtain live GFP-positive epidermal ESCs (eESCs) from *ecto-GFP* transgenic polyps [37] (*see* Subheadings 3.1 and 3.2). (**b**) Gating strategy: The cells are first gated based on the forward scatter (FSC-H) and side scatter (SSC) properties (gate A). Next, the intact, viable cell population is selected based on the intensity of Draq7 fluorescence, detected on the FL4-Area channel (gate B). Subsequently, the GFP-positive cells are identified considering the GFP fluorescence collected on the FL1 channel (gate C) and sorted after exclusion of cell doublets, based on the area and height of the FSC signal (gate D). (**c**) Analysis of the enrichment score of the GFP+ eESCs after re-running the sorted cells on the flow cytometer (FACS-2). Note that 96.6% of sorted cells are viable (gate B) and 99.5% are GFP-positive (gate C). The sorting was done with a Biorad cell sorter S3. (**d**) Microscopical control of the enrichment of the GFP+ eESCs after sorting. Green fluorescence (left panel) and bright field (right panel) images were acquired with a Leica DM5500 fluorescence microscope 3 h after sorting. Arrows point to cells with low GFP fluorescence. Scale bars: 75 μm

content that doubles during S-phase and is divided by twofold at the end of the G2-phase upon mitosis. The DNA content can be assessed quantitatively from staining with propidium iodide (PI), a DNA intercalating dye whose fluorescence is enhanced upon insertion between the bases [45]. This method is widely used to estimate the relative distribution of the cells between the G0/G1, S, or G2/M phases of the cell cycle, as shown in mammalian [46] or invertebrate cells from *Drosophila* [47], planarians [48], or *Daphnia* [49]. The DNA staining protocol presented here integrates a fast dissociation step of the tissue with trypsin–EDTA enzymatic digestion, followed by PI DNA labeling in a hypertonic staining solution in the presence of detergents [50]. This method allows the successful analysis of very small tissue fragments and the processing of a large number of samples in parallel while obtaining good quality DNA histograms as shown by the good coefficient of variations (CV) measured across the G0/G1 peak [6] (Fig. 4).

As an alternative, the monitoring of cell proliferation with the click-iT EdU detection assay was developed by Invitrogen. Similar to BrdU, EdU (5-ethynyl-2′-deoxyuridine) is a thymidine analog that gets incorporated into the newly synthesized DNA chain during the replication phase. In contrast to the antibody-based BrdU detection, the click-iT reaction consists of the chemical detection of EdU after a 30-min reaction between EdU and an Alexa photostable dye catalyzed by copper [51, 52]. This method, which can provide a dynamic view of the progression of DNA replication during the S-phase when several time-points are compared, is faster and easier than BrdU immunodetection because neither DNA denaturation nor immunodetection are needed. However, in *Hydra*, the cellular absorption of EdU in intact animals is low when compared to BrdU, and the number of S-phase cells detected with the click-iT EdU proliferation kit is not reliable. During regeneration, the tissues of amputated animals absorb well the thymidine analogue, and the EdU labeling procedure is well suited for monitoring cell proliferation (Fig. 5).

In this chapter, we report two distinct flow cytometry procedures to detect the cell cycle distribution in homeostatic or regenerative contexts. The first procedure analyses nuclei whose DNA is stained with PI after tissue dissociation while the second procedure relies on the pulse labeling of S-phase nuclei with EdU, a DNA-labeling process that takes advantage of the physiological replication process occurring in live animals. The protocols presented here rely on two distinct procedures for tissue dissociation that precedes flow cytometry. The relatively slow pronase dissociation is well suited to sort fluorescent cells by FACS, whereas the fast trypsin–EDTA dissociation is well suited for cell cycle analysis such as PI labeling or EdU click-iT labeling (Fig. 2). As this latter method discriminates between the cells in G1 or G2 from those in early or late S-phase, it provides a dynamic assessment of the

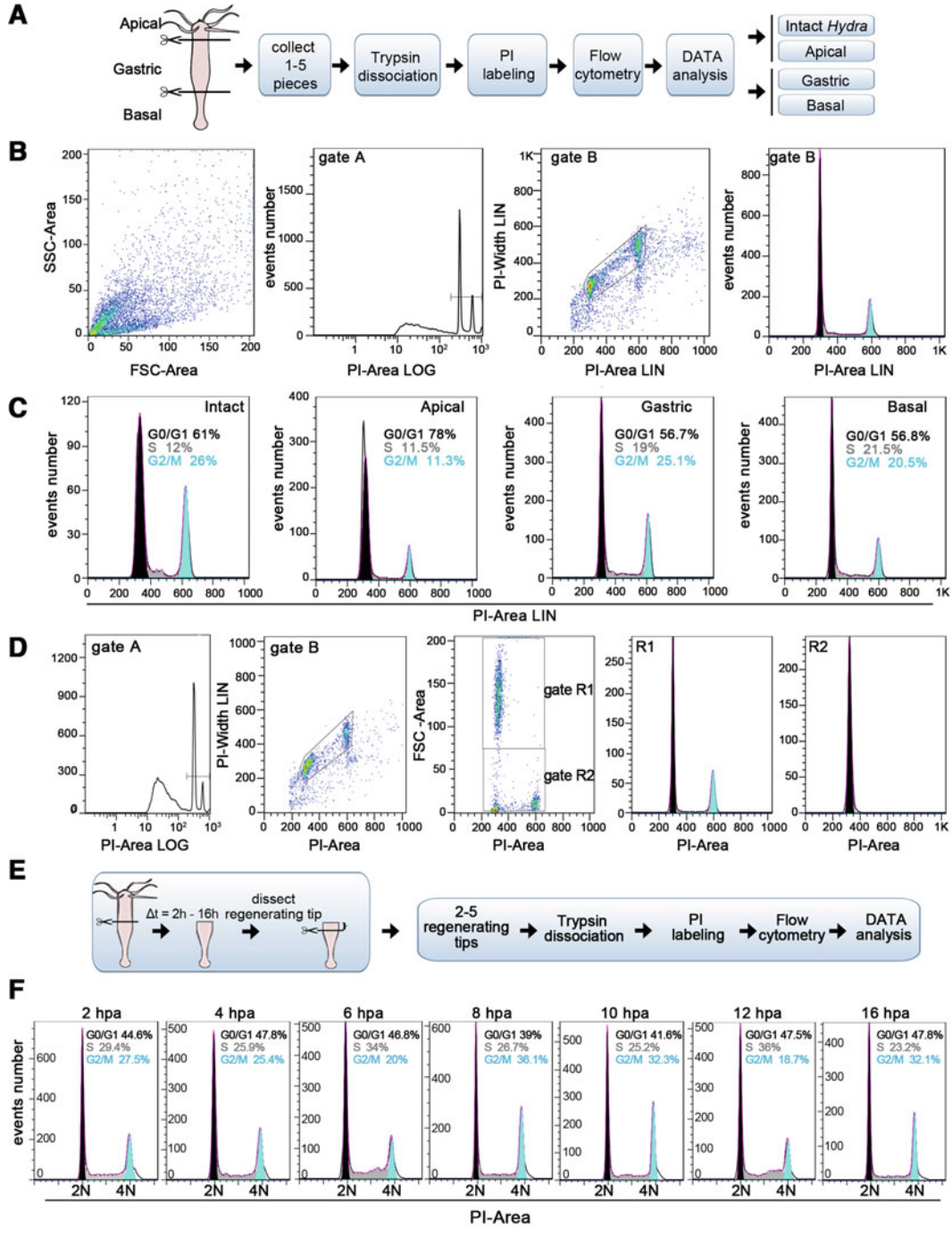

Fig. 4 DNA content measurement in *Hydra* cells after PI staining. (**a**) Workflow of the flow cytometry method used to analyze the cell cycle distribution after PI staining of DNA (*see* Subheading 3.3). (**b**) Typical successive gating procedure where the events are acquired based on the FSC/SSC parameters, then gated first on the channel that detects the PI fluorescence (gate A), followed by doublets and clumps exclusion after setting up a singlet discrimination PI-Area/PI-Width window (gate B). The cell cycle distribution (right panel) is deduced from the DNA content reflected by the intensity of the PI-Area signals of PI-labeled cells in gate B. (**c**) Cell cycle distribution in samples obtained after dissociation of whole animals (intact) or from different *Hydra* regions

number of cells progressing through the S-phase, when samples corresponding to different labeling periods are compared. In contrast, the PI labeling method, which is cheaper and faster, only provides a static view of the DNA content, allowing to deduce the cellular distribution between the different phases of the cell cycle at the time tissues were dissociated. Depending on the context and the experimental objective, these two flow cytometry methods are valuable tools for quantifying the rate of proliferating cells and the cell cycle progression during whole-body *Hydra* regeneration.

2 Materials

All stocks and working solutions are prepared with ultrapure, deionized MilliQ water in screw-capped bottles, sterilized either by autoclaving or filtration using a 0.22-μm filter. The bottles are stored at 4 °C or room temperature (RT) as indicated below. Check stock solutions regularly for any sign of contamination, discard them, and replace them with fresh ones when needed. To prepare the working solutions (1×), dilute any concentrated stock (10× or 20×) with ultrapure MilliQ water.

2.1 Dissociation of Hydra Live Cells

1. A transgenic *Hydra* strain constitutively expressing GFP (*see* **Note 1**).
2. Wild-type AEP *Hydra* strain.
3. 500× *Hydra* Medium Stock solution A: 60.57 g Tris–base–HCl in 900 mL H_2O, pH 7.7, bring to 1000 mL with H_2O. Sterilize by autoclaving, store at 4 °C for several weeks.

Fig. 4 (continued) (apical, gastric column, and basal). Note that the broader G0/G1 peak detected in samples from intact animals or apical region corresponds to a double G0/G1 peak formed by two different cell populations. (**d**) To identify these two distinct G0/G1 cell populations, a specific gating procedure was applied to the samples obtained after dissociation of tissues obtained from the apical region or from the whole animal. An additional gating based on the FSC-Area and PI-Area parameters allows the identification of these two populations as gate R1 and gate R2. Sorting of the nuclei from gate R1 revealed that this population is mainly composed of terminally differentiated nematocytes arrested in the G0 phase (data not shown), which are mainly found in the apical region. (**e**) Scheme depicting the flow cytometric procedure applied to head-regenerating tips (*see* Subheading 3.3). Animals bisected at the mid-gastric level were allowed to regenerate. At indicated time-points after bisection, the head regenerating tips were excised and prepared for analysis as shown in (**a**). (**f**) Cell cycle profiles measured in *H. magnipapillata* animals undergoing head regeneration. Five regenerating tips about 500 μm long were processed and analyzed as above. Note the increase in S-phase cells between 4 and 6 h post-amputation (hpa), followed by an increase in the G2 population at 8 hpa as previously reported [20]. The samples were run on a Gallios flow cytometer (Beckman Coulter) (**b–d**) or a BD FACSCalibur (**f**), and the data were analyzed with the FlowJo software and subjected to Watson's mathematical model to calculate the proportion of cells in each phase of the cell cycle

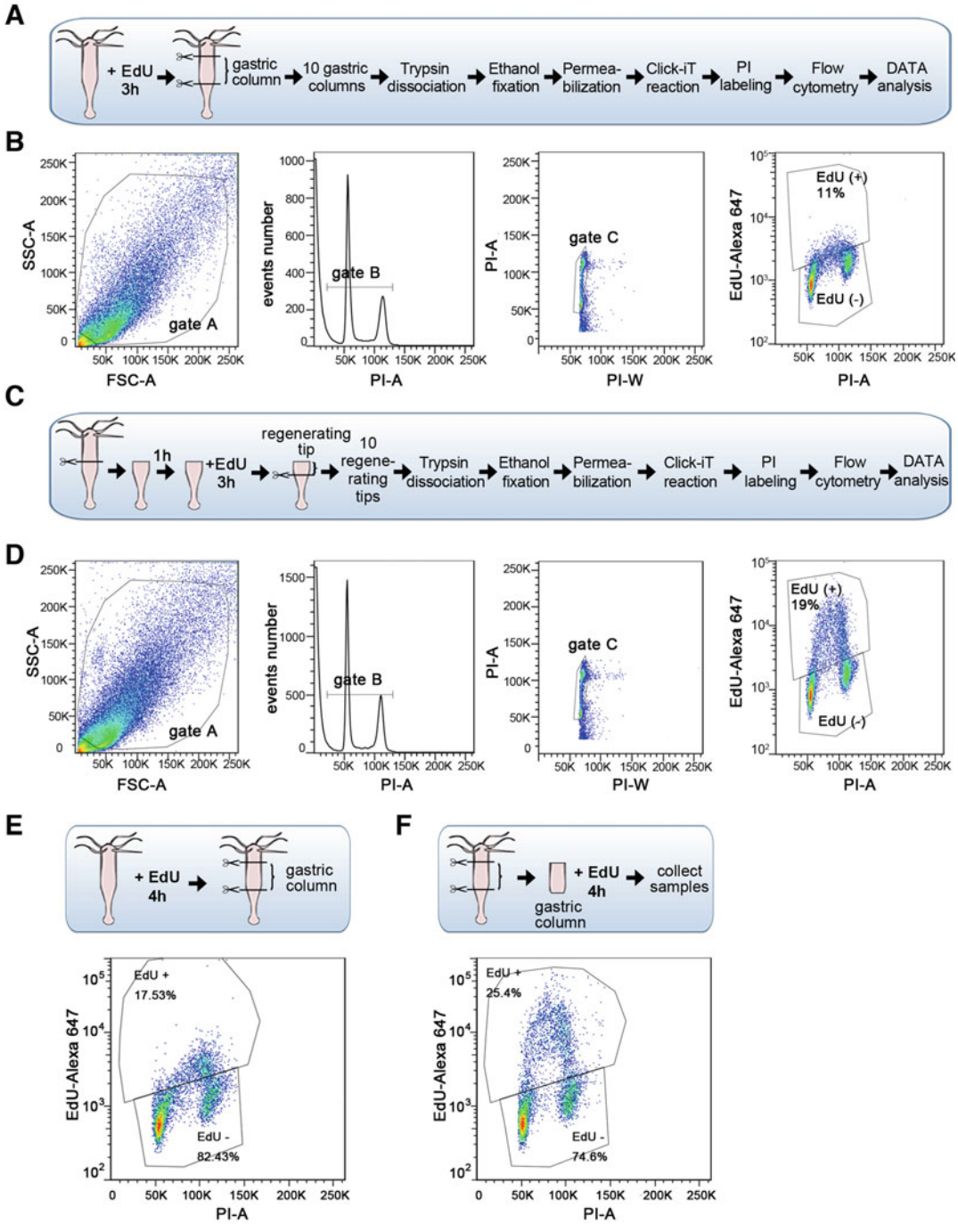

Fig. 5 Flow cytometry analysis of DNA replication in EdU-labeled Hydra cells. (**a**, **c**) Scheme illustrating the workflow of the flow cytometry procedure performed on samples obtained after dissociation of intact (**a**) or head-regenerating polyps (**c**) taken 4 h after bisection and incubated in EdU (5 mM) for 3 h. (**b**, **d**) To analyze cell proliferation, the cells were labeled with the click-iT EdU-Alexa 647 kit, the samples were run on a Fortessa flow cytometer, and the data collected based on the FSC and SSC signals (gate A). Next, the debris were removed from gate A by applying a second gating based on the PI-Area (PI-A) signal. The singlet cells were separated in a PI-Width (PI-W) and PI-A window, which helps to exclude the doublets (gate C). Finally, the

4. 500× *Hydra* Medium Stock solution B: 54.77 g $CaCl_2$•$6H_2O$, 14.6 g NaCl, 1.85 g KCl in 400 mL H_2O. Bring to 500 mL with H_2O. Sterilize by autoclaving, store at 4 °C for several weeks.
5. 10,000× *Hydra* Medium Stock solution C: 61.6 g $MgSO_4$•$7H_2O$ in 200 mL H_2O. Bring to 250 mL with H_2O. Sterilize by autoclaving, store at 4 °C for several weeks.
6. *Hydra* medium (HM): 2 mL stock solution A, 2 mL stock solution B, 100 µL stock solution C, 996 mL H_2O (*see* **Note 2**).
7. Dissociation medium (DM): 1.08 g KCl, 5.256 g $CaCl_2$•$6H_2O$, 1.184 g $MgSO_4$•$7H_2O$, 7.04 g sodium citrate, 2.748 g sodium pyruvate, 4.32 g glucose, 20 g TES-NaOH, pH 6.9 in 3.8 L H_2O. Bring to 4 L with H_2O, filter-sterilize, store at 4 °C (*see* **Note 3**).
8. Pronase E: 10 mg pronase E in 2 mL DM (*see* **Note 4**). Use immediately.
9. Membrane impermeable DNA dye (e.g., 0.3 mM Draq7, Biostatus).

2.2 PI Nuclei Staining for Cell Cycle Analysis

1. Trypsin–EDTA solution: 40 mg KCl, 6 mg KH_2PO_4, 35 mg $NaHCO_3$, 0.8 g NaCl, 9 mg Na_2HPO_4•$7H_2O$, 20 mg EDTA•4Na•$2H_2O$, 1 mg Phenol Red, 50 mg trypsin in 90 mL H_2O. Bring to 100 mL, filter-sterilize, aliquot, store at −20 °C (*see* **Note 5**).

Fig. 5 (continued) cells selected from gate C were analyzed in a PI/ EdU-Alexa Fluor 647 dot-plot area, where the negative and positive EdU gates are defined by the fluorescence intensity in the EdU-Alexa 647 channel. Thus, cells in S-phase, which have incorporated EdU, form the EdU(+) population, while cells in G0/G1 or G2/M correspond to the EdU(−) population. Note the lower percentage of EdU(+) cells and the lower fluorescence intensity on the Alexa 647 channel when intact animals (**b**) were incubated with the EdU solution compared to the regenerating ones (**d**). This result indicates that EdU incorporation is lower in gastric cells when intact *Hydra* are exposed to EdU compared to the regenerating ones. Beside a regulation linked to regeneration, this difference might actually be artefactual, reflecting the barrier effect of the *Hydra* cuticle to EdU in intact animals when compared to the wounded ones. This hypothesis is supported by the fact that the observed proportion of EdU-labeled cells (11%) after a 3-h labeling is lower than that measured by microscopic analysis after BrdU incorporation or by flow cytometry after PI staining method [6, 17, 20, 25, 26]. Indeed, in starved conditions, about 20% of the cells are cycling in gastric tissues from intact animals (*see* Fig. 4c). (**e**, **f**) Comparison between two experimental conditions: one where intact animals were exposed to EdU for 4 h before the central gastric tissue is dissected (**e**), another where the extremities of the animal are first amputated and then the severed gastric pieces are incubated in EdU for 4 h (**f**). After washing, the samples from both experiments were treated as described in Subheading 3.4. Analysis of the EdU-Alexa 647/PI-A data graph (**a** and **b**, lower panels) shows that the percentage of EdU-Alexa 647-positive cells is 50% higher when the central gastric pieces are amputated before EdU incubation compared to that obtained in gastric pieces amputated after EdU incubation. Note also that prolonging EdU incubation improves the fraction of EdU (+) cells: 17.5% after 4 h vs. 11% after 3 h

2. Fetal bovine serum (FBS) from commercial sources. Aliquot, store at −20 °C.
3. 10× PBS stock solution: 80 g NaCl, 2 g KCl, 29 g Na_2H-PO_4•$12H_2O$, 2 g KH_2PO_4 in 900 mL H_2O, pH 6.8. Bring to 1000 mL with H_2O. Sterilize by autoclaving, store at RT (*see* **Note 6**).
4. EDTA solution: 0.931 g EDTA in 40 mL H_2O, pH 8. Bring to 50 mL with H_2O. Filter-sterilize, store at RT.
5. Tris–HCl solution: 0.606 g Tris–base–HCl in 90 mL H_2O, pH 8, bring to 100 mL with H_2O. Sterilize by autoclaving, store at 4 °C.
6. Propidium iodide (PI) solution: 25 mg PI in 25 mL H_2O. Store at 4 °C up to 1 year protected from light (*see* **Note 7**).
7. RNase A solution: 200 mg RNase A in 10 mL Tris–HCl 50 mM. Store at 4 °C.
8. Hypertonic NP-40 buffer: 10 mL 10× PBS, 0.5 mL Nonidet NP-40 substitute, 100 µL 0.5 mM EDTA in 89.4 mL H_2O, Filter-sterilize, store at 4 °C (*see* **Note 8**).
9. Labeling solution (LB): 140 µL PI solution, 35 µL RNAse A solution in 2.8 mL hypertonic NP-40 buffer. Prepare freshly, keep on ice until use (*see* **Note 9**).

2.3 Click-iT EdU-Detection of Proliferating Hydra Cells

1. Click-iT Plus EdU Flow Cytometry Assay kit (C10634) (*see* **Note 10**).
2. 2× 5-ethynyl-2′-deoxyuridine (EdU) solution: 25.23 mg EdU, 10 µL DMSO in 10 mL HM. Prepare freshly (*see* **Note 11**).
3. 1× EdU solution: 1.5 mL of 2× EdU solution, 1.5 mL HM. Enough for one experimental condition, increase accordingly.
4. 1× PBS: 50 mL 10× PBS stock solution, 450 mL H_2O (*see* **Note 6**).
5. 1% Bovine albumin serum (BSA) solution: 1 g BSA in 100 mL 1× PBS. Filter-sterilize, store at 4 °C for up to 1 week (*see* **Note 12**).
6. 100% cold ethanol: 50 mL absolute ethanol, store at −20 °C.
7. Permeabilization and wash buffer (PWB): 5 mL Component E (C10634), 45 mL 1% BSA solution. Store at 4 °C for 1 week.
8. 10× buffer additive stock: 400 mg Component G (C10634) in 2 mL H_2O. Aliquot, store at −20 °C.
9. 1× buffer additive: 5 µL of 10× buffer additive stock, 45 µL H_2O for one sample. Prepare freshly. Multiply by the number of desired samples plus one.

10. Click-iT EdU detection cocktail: 10 μL Copper protectant (component F), 2.5 μL fluorescent Alexa picolyl azide (component B), 50 μL 1× buffer additive, 438 μL 1× PBS. Use within 15 min.
11. DNA staining solution: 12.5 μL PI solution, 5 μL RNase A, 483 μL PWB for one sample. Multiply by the number of desired samples plus one.

2.4 Equipment

1. Stereomicroscope (for example Olympus SZX10 with a 1.25× objective).
2. Pasteur glass pipette.
3. Borosilicate Pyrex 25 × 20 cm rectangular dishes (Pyrex dishes for alimentation).
4. Surgical scalpel No. 3.
5. Surgical blades No. 10.
6. Non-coated plastic petri dish.
7. Silicon bulbs, 5 mm diameter for Pasteur pipette.
8. Mini cell strainer, 70-μm mesh size.
9. Nylon mesh 100-μm pore size.
10. 5-mL polystyrene tubes for flow cytometry.
11. Digital dry bath.
12. Steriflip and steritope (0.22 μm) for filtration.
13. Mini vacuum pump.
14. Flow cytometer equipped with appropriate lasers.
15. Cell sorter equipped with 488 and 561 nm lasers.

3 Methods

3.1 Live Hydra Cell Suspension

1. Collect 250 GFP-expressing transgenic animals with the help of a glass Pasteur pipette (*see* **Note 13**) in a Pyrex dish.
2. Collect 50 wild-type *H. vulgaris AEP* non-transgenic animals with the help of a glass Pasteur pipette in a plastic petri dish.
3. Wash the animals with HM.
4. Check the green fluorescence at the stereomicroscope.
5. Removes animals with a weak or mosaic fluorescence.
6. Dispatch the transgenic animals in five plastic petri dishes (9 cm diameter), 50 animals per dish.
7. Wait 1–2 min for the animals to relax under the light and extend at their maximal length (*see* **Note 14**).

8. Cut with a scalpel the animals of a given dish twice to remove both the apical and the basal part (Fig. 3a).
9. Collect the remaining 50 central body columns in a 1.5-mL tube.
10. Remove as much as possible of the HM liquid from the 1.5-mL collection tube.
11. Add 400 µL of pronase E solution to the collection tube (*see* **Note 15**).
12. Transfer the tube to ice.
13. Repeat **steps 7–12** to cut the animals of all the dishes.
14. Carefully pool all the gastric regions into one tube using a clean Pasteur pipette.
15. Repeat **steps 7–12** with 50 wild-type AEP animals.
16. Transfer the tubes to RT.
17. Leave the samples to dissociate for 1 h.
18. Mix the samples by gently pipetting up and down several times.
19. Wait for 15 min.
20. Repeat **steps 18** and **19** three more times.
21. Centrifuge the samples at 100 rcf for 5 min at 4 °C.
22. Gently replace the supernatant with 1 mL of DM.
23. Slightly resuspend the pellet by pipetting up and down several times.
24. Re-centrifuge the samples at 100 rcf for 5 min at 4 °C.
25. Remove the supernatant and resuspend the pellet in 3 mL DM (*see* **Note 16**).
26. Filter the cell suspension using a mini PluriStrainer of 70-µm pore diameter (*see* **Note 17**).
27. Collect the filtrate in a 15-mL tube.
28. Dilute 15 µL of filtrated cell suspension into 135 µL of DM.
29. Load 10 µl of the diluted cells in each chamber of a hemocytometer.
30. Count four squares from the upper and lower chambers.
31. Calculate the cell density (number of cells/mL) by multiplying the obtained average cell number with 10^5.
32. Adjust the cell density to 10^6 cells/mL with DM.
33. Keep the samples on ice until sorting.

Table 1
Basic terminology used in this chapter related to flow cytometry and cell cycle analysis

Glossary	
Event	A single particle or cell detected by the flow cytometer
FL1	The fluorescence detected and amplified by the photomultiplier tube (PMT) FL1, in the green range of the spectrum (500–565 nm)
FL1-A	The area of the electric pulse (signal) generated by the PMT corresponding to FL1
FL2	The fluorescence detected and amplified by the PMT FL2, in the yellow range of the spectrum (565–590 nm)
FL3	The fluorescence detected and amplified by the PMT FL3, in the orange range of the spectrum (590–625 nm)
FL4	The fluorescence detected and amplified by the PMT FL4, in the red range of the spectrum (625–700 nm)
FL4-area	The area of the electric pulse (signal) generated by the PMT corresponding to FL4
FSC (forward scatter)	This measurement discriminates cells or nuclei according to their size based on the light scattered at an angle of less than 10° as the cells cross the laser beam [62, 63]
FSC-area	Area of the FSC signal
FSC-height	Height of the FSC signal
GATE	A frame or outline that delimitates a subset or sub-population of events. This boundary is drawn by the experimenter on the graph representing the data set [63]
GATING	The process of plotting a gate, the boundaries of a set of events on a diagram or histogram [63]
LIN	Linear scale
LOG	Logarithmic scale
PI-width	Width of the peak signal generated by the PI fluorescence detected on FL2 or FL3 according to the flow cytometer
PI-area	Area of the peak signal generated by the PI fluorescence detected on FL2 or FL3 according to the flow cytometer
SSC (side scatter)	This measurement discriminates cells or nuclei according to their granularity based on the light scattered at an angle of 90° as the cells cross the laser beam [62, 63]

3.2 Flow Cytometry Sorting of Hydra GFP-Expressing Stem Cell (FACS Protocol)

1. Replace the sheath fluid from the FACS sorter with DM (*see* **Note 18**).
2. Add Draq7 to the cell suspension to a final concentration of 0.3 μM.
3. Incubate for 10 min at RT (*see* **Note 19**).
4. Set up the gating windows by selecting first the viable cells from the debris (FSC-H/SSC-H window, Table 1), next the Draq7-negative cells, then the GFP-positive population and finally by excluding the cell doublets among the GFP-positive stem cells (Fig. 3b).

5. Collect the GFP fluorescence on the corresponding detector with a 525/30 nm band pass filter and the Draq7 fluorescence on the far red detector with a 655 long pass filter (Fig. 3b).
6. Set the cell-sorting rate at 1500 cells/s and use a 100-μm nozzle to pass the cells. On the Biorad S3 sorter, the pressure is set up at 20 psi. These cell-sorting conditions were optimized for a density of 1×10^6 cells/mL (*see* **Note 20**).
7. Add 60 μL of DM into a 1.5-mL collection tube (*see* **Note 21**).
8. Sort the GFP-positive stem cells into the tube filled with DM.
9. Check the enrichment factor by diluting 25 μL of sorted cells into 275 μL of DM and re-running the sample again on the sorter (Fig. 3c, *see* **Note 22**).
10. Centrifuge the tubes with the sorted cells at 100 rcf for 5 min at 4 °C.
11. Resuspend the pellet in the requested buffer for the desired application (RNA extraction, proteomics, etc., *see* **Note 23**) or in labeling buffer (LB) to proceed with cell cycle measurements by starting at **step 15** in Subheading 3.3.

3.3 Cell Cycle Measurement: PI Staining of Trypsin-Dissociated Cells

1. Place up to 20 animals in a 10-cm plastic petri dish pre-filled with 50 mL HM for each regenerating time point.
2. Wait 1–2 min to let the animals to relax and extend at their maximal length under the light.
3. When different body regions are analyzed, cut the animals to the desired level (Fig. 4a) using a scalpel (*see* **Note 14**) and continue with **step 8**.
4. For regeneration experiment, cut the animals at mid-gastric position (Fig. 4e).
5. Transfer the lower halves that will regenerate their head to a new pre-filled dish with HM.
6. Let the animals regenerate at 18 °C for 0–72 h.
7. Cut off at selected time-points the regenerating tip (about 200 μm thick) from two to five animals (Fig. 4e).
8. Collect the regenerating tips into a 1.5-mL tube.
9. Repeat **steps 7** and **8** at least three times to be able to analyze at least three replicates per time-point (*see* **Notes 24** and **25**).
10. Remove the maximum amount of HM with a micro-pipette from each tube.
11. Add 50 μL of Trypsin–EDTA solution to each tube.
12. Heat the tube for 5 min at 37 °C in a block-heater.
13. Dissociate the tissue by gently mixing the samples about 20 times up and down with a micropipette (*see* **Note 26**).

14. Add 20 μL of FBS and place the tube on ice (*see* **Note 27**).
15. Repeat **steps 10–14** to dissociate the next tube.
16. Add 430 μL of LB (*see* **Notes 28** and **29**).
17. Incubate the samples for 30 min in the dark at RT (*see* **Note 30**).
18. Transfer the samples into 5-mL flow cytometry tubes and store them on ice protected from light.
19. Run the samples on a regular flow cytometer using a low flow rate (*see* **Note 31**).
20. Set up the threshold on the PI channel and detect the PI fluorescence after linear amplification on the corresponding channel according to your flow cytometer (FL2 or FL3) as shown in Fig. 4 (*see* **Note 32**).
21. Gate the nuclei by separating the debris from the PI-labeled nuclei (PI-Area (LOG)/Events number) and by excluding the doublets and clumps (PI-Area (LIN)/PI-Width (LIN)) (Fig. 4b, *see* **Note 33**).
22. Acquire between 10,000 and 20,000 events for each sample.
23. Analyze the cell cycle profiles by using the software either available on your flow cytometer or commercially available such as FlowJo (*see* **Note 34**).
24. Clean the flow cytometer according to the washing/cleaning procedures of the supplier (*see* **Note 35**).

3.4 Cell Cycle Measurement: Click-iT EdU Labeling of Hydra Cells Replicating Their Genomic DNA (S-Phase)

1. Follow **steps 1** and **2** in Subheading 3.3.
2. Cut 10–15 animals at the mid-gastric level (Figs. 4e and 5c).
3. Collect the regenerating halves in a 2-mL tube.
4. Adjust the volume to 1 mL with HM.
5. Add 1 mL of 2× EdU solution.
6. Transfer the animals into a 12-well plate.
7. Add 3 mL of 1× EdU solution to each well containing animals (*see* **Note 36**).
8. Let the animals regenerate for the required time.
9. Transfer the animals into a 1.5-mL tube at the selected time-points.
10. Wash out the EdU solution by replacing five times the medium with fresh HM.
11. Follow **steps 10–13** in Subheading 3.3.
12. Add 20 μL of FBS and 650 μL of PBS to each tube.
13. Centrifuge the samples at 350 rcf, 5 min at 4 °C.

14. Gently aspire the supernatant without touching the sediment with the help of a pipette connected to a vacuum pump.
15. Add 200 µL of PBS to each tube.
16. Gently resuspend the pellet.
17. Add drop by drop 800 µL of pre-chilled 100% ethanol while vortexing the tubes at medium speed.
18. Fix the samples on ice for 1 h and overnight at 4 °C.
19. Centrifuge the samples next day at 650 rcf for 5 min at 4 °C.
20. Resuspend the pellet in 1% BSA.
21. Transfer the samples in a 5-mL flow cytometry tube.
22. Repeat **step 19**.
23. Resuspend the pellet in 1 mL of PWB.
24. Incubate the samples at RT for 15 min.
25. Follow **step 19**.
26. Gently resuspend the sample in 100 µL of PWB.
27. Add 500 µL of click-iT EdU detection cocktail to each tube.
28. Mix gently.
29. Incubate the tubes in the dark for 30 min (*see* **Note 37**).
30. Add 1 mL of PWB to each tube.
31. Re-centrifuge the tubes as in **step 19**.
32. Resuspend the pellet in the DNA-staining solution.
33. Incubate the samples at RT for 30 min protected from light.
34. Prepare two control tubes: one with cells only exposed to the DNA-staining solution and another with cells only exposed to the click-iT EdU detection cocktail (*see* **Notes 30** and **38**).
35. Filtrate the samples into a new flow cytometry tube using a cell strainer (70-µm mesh).
36. Collect the EdU-Alexa dye fluorescence according to the selected Alexa fluorochrome, on the corresponding detector: here the Alexa 647 dye was collected on a 660/20 band pass filter.
37. Detect the PI fluorescence after linear amplification on the corresponding channel according to your flow cytometer (FL2 or FL3) as shown in Fig. 5.
38. Set up the gating windows (Fig. 5b): select first the cells based on the FSC/SSC parameters, remove the debris by gating the cells labeled with PI (PI-A/events number histogram), remove the doublets by gating singlet cells in a PI-W/PI-A window, open a PI/EdU-Alexa 647 dot-plot.

39. Analyze the data with available software and estimate the percentage of EdU-positive cells that correspond to the proliferating population based on the EdU-Alexa 647 fluorescence intensity (Fig. 5b, d–F).

4 Notes

1. In the protocol described here, we used the *ecto-GFP* strain [37], a transgenic line produced in the laboratory of T. Bosch (Kiel, Germany) that constitutively expresses GFP in the epidermal epithelial stem cells. In a previous work [40], we also applied flow cytometry to *Hydra* transgenic strains that express GFP in gastrodermal epithelial cells from the *endo*-GFP line [38] or interstitial cells from the *cnnos1*-GFP strain [39]. These strains can be obtained from the Transgenic Hydra Facility at the University of Kiel (http://transgenic-hydra.org).
2. Here the *Hydra* medium is prepared according to [53].
3. The dissociation medium (DM), is a hyperosmotic solution (70 mOsm) used to dissociate the *Hydra* polyps and obtain live cells, which was initially used for reaggregation studies [54]. The protocol indicates that it is necessary to prepare a large volume of DM (4 L), as DM is also used to replace the classical fluid sheath in the sorter (*see* **Note 18**).
4. Pronase E is a mixture of several proteases obtained from *Streptomyces griseus*; it can be stored as powder at −20 °C. Select a product that is similar to P6911 from Sigma and has an enzymatic activity higher than 4 U/mg (the P6911 product is unfortunately discontinued).
5. The indicated trypsin–EDTA solution is used for mammalian cell culture. Therefore, any commercially available solution with the indicated concentration can be used to dissociate *Hydra* tissue.
6. It is not necessary to adjust the pH of the 10× PBS stock solution, because after dilution to 1× PBS, the pH reaches 7.4.
7. Always wear gloves when working with PI as PI is a DNA intercalating agent with mutagenic properties. Dispose the PI wastes according to the safety procedure established in your lab.
8. Nonidet NP-40 substitute is a viscous detergent that must be carefully aspirated during pipetting. Gently stir the labeling buffer to avoid foaming and bubbles.
9. The indicated PI and RNase-A concentrations correspond to the final concentration obtained after mixing the dissociated *Hydra* cells with the labeling buffer.

10. Different Click-iT Plus EdU Flow Cytometry kits have been developed by Invitrogen, which only differ by the Alexa fluorophores that have distinct spectral properties among the kits. Choose the right combination for the laser equipment available on your flow cytometer. The kit used here (Click-iT plus Alexa Fluor 647 flow cytometry assay kit, ref. C10634) is compatible with the detection of cell cycle dyes, GFP and mCherry. The protocol can be adapted to monitor the proliferation of a population of fluorescent reporter-expressing cells.
11. EdU is a potentially hazardous agent because it readily gets incorporated into the genome. It must therefore be handled with care while wearing gloves. To increase the solubility and uptake of EdU by *Hydra* cells, it is necessary to add DMSO to the EdU solution, however at a concentration not exceeding 0.5%, as DMSO above 1% affects the organization of epithelial cells [55].
12. To prepare the BSA solution, first transfer the powder to a 50-mL tube and then add the required volume of PBS. Mix the solution by gentle stirring to avoid foaming and bubble formation.
13. The number of transgenic animals required to isolate GFP-positive cells should be defined according to the expected number of GFP-positive cells obtained after sorting. We usually obtain 10^5 GFP-positive sorted cells from 100 gastric columns. Avoid taking animals that have been fed on the same day, as the gastrodermal epithelial cells would be full with digestive vacuoles, fragile, and thus easy to break. Moreover, the content of those digestive vacuoles is labeled by nuclei dyes, thus increasing the debris and noise level in the measurements.
14. To cut the animal at the correct position along the axis, let the animals relax under the stereomicroscope for about a minute. Apply the scalpel perpendicular to the body column and cut quickly. Change the blade regularly as it rusts easily.
15. *Hydra* epithelial cells are highly adhesive and re-aggregate rapidly after tissue dissociation [54]. The formation of clumps is a serious problem for flow cytometry as the clumps can clog the nozzle. For this reason, we prefer enzymatic dissociation with pronase-E than mechanical dissociation as initially established by Gierer and colleagues in 1972. The enzymatic method allows mesoglea lysis and tissue disintegration, producing viable cells that have lost their adhesiveness [56].
16. *Hydra* epithelial stem cells are large cells with a cuboidal or columnar shape, and they show a large cytoplasm and a high cytoplasm to nucleus ratio. They are highly sensitive to centrifugal forces; therefore, the centrifugation steps should be performed at a low speed to prevent their disruption. By contrast,

ISCs are much smaller than ESCs, and they show a higher nuclear to cytoplasmic ratio and a better resistance to centrifugation [56]. If ISCs are considered for sorting, an additional centrifugation step at 300 rcf is requested.

17. Always filtrate the cell suspension to separate the undigested small tissues fragments that can block the tubing or nozzle. For the filtration of volumes greater than 1 mL, use a 70-μm Pluristrainer. For small volumes, a homemade filter can be manufactured with a nylon mesh with a porosity of 70–100 μm. Use a plastic conical tip for 1-mL pipette, cut it about 1 cm from the top, cover the tip with a 1.5 × 1.5 cm piece of nylon mesh and insert it into a new intact conical tip. Transfer the cell suspension into the sectioned tip, firmly insert the micropipette, then gently press the micropipette plunger resulting in filtering the cell suspension through the nylon mesh, collecting the filtrate into a new tube.
18. The ProFlow sheath fluid, used in the flow cytometer fluidics to transport and focus the samples in the flow chamber, is usually the saline solution PBS, which is isotonic for mammalian cells but highly hypertonic for *Hydra* cells given their low osmolarity, lower than 10 mOsm [54, 57, 58]. Consequently, to prevent drastic shrinkage of the *Hydra* cells by water leakage, PBS should be replaced by DM, which has a much lower osmolarity (70 mOsm) than PBS (285–315 mOsm). The DM medium was previously tested in flow cytometry on beads (Spherotec) and the eight sorted peaks were found to be perfectly pure. Therefore, DM ensures the correct hydrodynamic focusing, the correct formation of the core stream, and the deflection of the droplets in the sorting flow chamber.
19. Draq7 is a nuclear far-red fluorescent dye that labels only dead or permeabilized cells as it cannot enter intact live cells. As consequence, Draq7 staining allows to exclude the damaged cells and to sort only viable cells.
20. Sorting conditions should be adjusted to run about 1×10^3 cells/s, and optimal conditions are depending on the initial density of the cell suspension. Depending on the available cell sorter, the pressure of the fluid sheath must be set at a minimum level so as not to damage the integrity of the cells. The best balance must be established between cell density, flow pressure, and sorting time.
21. The size of the collection tube and the amount of recovery medium should be adapted to the expected number of sorted cells and the objective of the experiment. According to our experience, on a Biorad S3 sorter, 1×10^5 cells are separated in about 400 μL of medium. If a higher cell yield is required ($4–5 \times 10^5$ cells), use larger tubes such as 5-mL flow cytometry

tubes or similar tubes available for your sorter. For RNA extraction, a high RNA yield (1 mg) was obtained with the minikit RNAeasy Plus (Qiagen) from 3×10^5 sorted cells.

22. The level of enrichment of GFP-positive cells among the sorted cells can be tested by re-running the sorted samples on the flow cytometer. In our hands, the re-analyzed samples are viable and the percentage of Draq7-positive cells is low (Fig. 3c). These Draq7-positive cells have a damaged cell membrane and show an intermediate GFP fluorescence profile, probably because cytoplasmic GFP can leak. An example of low GFP and broken membrane cells is shown in Fig. 3d, where the re-sorted cells were imaged under a fluorescent microscope. The sorted samples can also be analyzed with the Tali image-based cytometer (Invitrogen), which indicates the size and viability of the sorted cells.
23. To avoid damage to the sorted cells, process the samples quickly for the desired application. If transcriptomic analysis is planned, resuspend the cells in RNA extraction buffer and, if possible, process them immediately according to the supplier's instructions. Alternatively, the cells can be resuspended in the RNA protect Cell Reagent (Qiagen) and stored for a short period before RNA extraction.
24. The head-regenerating tip is the area located immediately below the bisection plan. It regenerates the head and is about 200 μm long. It is important to allow the animals to relax for a few minutes before sectioning to properly estimate the size of the slice to be cut. Indeed, cells behave differently in the head-regenerating tip than in the underlying tissue [25], and amputation of contracted polyps leads to the removal of larger slices where the regenerating tissue is diluted with homeostatic tissue.
25. The presented procedure (Fig. 4c, f) is adapted to allow the analysis of the cell cycle profile from very small tissue fragments containing a low number of cells (10^4) up to large tissue samples comprising 5×10^5 cells, corresponding to four or five medium-sized animals [6]. If a larger number of cells is to be analyzed, the volume of the labeling solution should be increased to adjust the cell density to a maximum of 1×10^6 cells/mL.
26. The established procedure (Fig. 4c, f) is based on a quick and easy method of tissue dissociation, which combines enzymatic dissociation by trypsin–EDTA with mechanical rupture. This method ensures complete dissociation of *Hydra* tissues, including the mesoglea and clusters of nematoblasts [6].

27. FBS contains protease inhibitors such as alpha 1-antitrypsin and is routinely used in mammalian cell culture to inactivate trypsin activity [59].
28. Incubation in the DNA-staining solution, which is hypertonic to *Hydra* cells and contains a substitute for NP-40 detergent, induces complete rupture of cell membranes [50]. As a result, this method provides mainly nuclei and very few cell clusters, which reduces sample preparation time since there is no need to filtrate the samples prior to flow cytometry.
29. The quality of the samples is determined by a low coefficient of variation (CV) across the G0/G1 peak. If too high, the accuracy of the measurement is limited. One parameter that can influence the CV is the stoichiometry of DNA labeling with PI. To ensure homogenous DNA staining, excess PI should be added. The concentration of 50 μg/mL has been shown to be appropriate for efficient labeling of different cell types and for a maximum cell density of 2×10^6 cells/mL [60].
30. PI is a DNA intercalating dye that binds to both DNA and double-strand RNA. Therefore, the labeling solution must contain RNase-A (100–200 μg/mL), which digests the RNA present in the sample. An incubation step of up to 30 min at RT is sufficient to degrade the RNA; subsequently, the tubes can be kept on ice for up to 2 h without altering the quality of the samples.
31. When the DNA content is measured by flow cytometry, the flow rate should be kept low, and the number of events should not exceed 200/s for optimal analysis of the sample with the best possible resolution (CV) of the PI fluorescence.
32. *Hydra* tissues contain about 12 different cell types that vary greatly in size and shape, with nuclei ranging from 5 to 15 μm [61]. To take into account this heterogeneity and to avoid the loss of small events/nuclei on the FSC channel, the threshold value should be set on the PI detector. In this way, only unwanted debris or noise is removed, and all intact nuclei stained with PI are acquired.
33. The name of acquisition parameters might slightly vary between the different types of flow cytometers. The acquisition should be done on the linear (LIN) and logarithmic scale (LOG) of the PI detector. The flow cytometry terms used in this chapter are presented in Table 1.
34. In general, CV values for samples taken from the central body column or from the basal region are below 3, which is acceptable given the heterogeneity of the *Hydra* tissue (Fig. 4c). However, we noted that when head region or intact animal is analyzed, a very wide G0/G1 peak is observed in the histogram (Fig. 4c). This wide peak actually consists of a double G0/G1

peak that corresponds to two different populations of nuclei. Therefore, to avoid misestimation of the S phase, we apply a different gating procedure to analyze the cell cycle profile of samples taken from the apical part of the animal (Fig. 4d), where the CV value did not exceed 3.0.

35. At the end of the acquisition, the flow cytometer must be cleaned immediately according to the protocol established in your facility, as PI is very adherent and persists in the tubing, contaminating subsequent acquisitions. Usually a 5 min wash with BDFACS Clean (bleach solution), followed by a 5 min wash with BDFACS Rinse (detergent solution) and a 5 min wash with H_2O is sufficient to clean the system.
36. The volume of the 5 mM EdU solution should be calculated considering that the standard conditions for regeneration are to maintain one regenerating animal in a minimal volume of 0.5 mL medium.
37. According to the supplier, the incubation time of 30 min with the click-iT EdU cocktail should not be exceeded.
38. Use unstained cells to set up the PMT voltage of each detector and use cells labeled with a single fluorophore to precisely adjust the voltage required for sample acquisition. If the emission spectra of DNA and Alexa dyes overlap, adjust the compensation voltage. The combination of Alexa Fluor 647 and PI does not require any compensation (Fig. 5b, d–f), since the spillover between the two fluorochromes is limited. However, if PI and Alexa Fluor 488 are selected, appropriate fluorescence compensations are required for correct sample acquisition.

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