Protocol

In Vivo Assessment of Neural Precursor Cell Cycle Kinetics in the Amphibian Retina

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Cell cycle progression is intimately linked to cell fate commitment during development. In addition, adult stem cells show specific proliferative behaviors compared to progenitors. Exploring cell cycle dynamics and regulation is therefore of utmost importance, but constitutes a great challenge in vivo. Here we provide a protocol for evaluating in vivo the length of all cell cycle phases of neural stem and progenitor cells in the post-embryonic *Xenopus* retina. These cells are localized in the ciliary marginal zone (CMZ), a peripheral region of the retina that sustains continuous neurogenesis throughout the animal's life. The CMZ bears two tremendous advantages for cell cycle kinetics analyses. First, this region, where proliferative cells are sequestered, can be easily delineated. Second, the spatial organization of the CMZ mirrors the temporal sequence of retinal development, allowing for topological distinction between retinal stem cells (residing in the most peripheral margin), and amplifying progenitors (located more centrally). We describe herein how to determine CMZ cell cycle parameters using a combination of (i) a cumulative labeling assay, (ii) the percentage of labeled mitosis calculation, and (iii) the mitotic index measurement. Taken together, these techniques allow us to estimate total cell cycle length (T_C) as well as the duration of all cell cycle phases ($T_{S/G2/M/G1}$). Although the method presented here was adapted to the particular system of the CMZ, it should be applicable to other tissues and developmental stages as well.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

1-butanol (100%)

5-ethynyl-2'-deoxyuridine (EdU) (Thermo Fisher Scientific)

Alexa-coupled secondary antibody (1 mg/mL) (e.g., Thermo Fischer Scientific)

Antifade mounting medium (e.g., FluorSave reagent [Thermo Fischer Scientific])

Anti-phospho-Histone H3 (PH3) antibody (1 mg/mL) (e.g., Millipore 06-570)

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From the *Xenopus* collection, edited by Hazel L. Sive.

Supplemental material is available for this article at cshprotocols.cshlp.org.

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Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot105536

Blocking solution (PBT with 10% goat serum)

Click-iT EdU Alexa Fluor imaging kit (Thermo Fisher Scientific)

Follow the manufacturer's instructions for preparing stock solutions.

Ethanol (100%)

Prepare 95%, 70%, and 50% solutions in 1× PBS.

Glycerin albumen (100% stock solution) (e.g., VWR)

Store stock solution at −20°C. Immediately before use, prepare a fresh 3% solution in dH₂O.

HCl (2 N) (optional, if using bromo-2'-deoxyuridine [BrdU] instead of EdU)

Hoechst 33342 fluorescent stain (5 μM)

Modified Barth's saline (MBS) <R>

Paraffin wax (e.g., 00403 HISTOWAX [Histolab])

Paraformaldehyde (PFA) (4%)

PBT (1× PBS, 0.2% Triton X-100)

Phosphate-buffered saline (PBS) (1×, pH 7.2–7.6)

Tricaine methanesulfonate solution (MS222) (Sigma-Aldrich) (0.4 g/L buffered to pH 7.0–7.5 with sodium bicarbonate)

Unmasking citrate solution (10 mM sodium citrate, 0.05% Tween 20 in dH₂O)

Xylene (100%)

Equipment

Coplin jars (glass)

Embedding molds (plastic, disposable)

Fluorescence microscope

Heating plate at 50°C

Humidified chamber

Image processing software (e.g., FIJI or Adobe Photoshop)

Incubators at 18°C–20°C, 37°C, and 60°C

Microscope slides and coverslips

Microtome (e.g., Microm HM 340E)

Parafilm or GelBond (Lonza)

Petri dishes

Pipettes (plastic)

Razor blades

Roller shaker

Vials (glass)

METHOD

For the principles of the methods described here for determining the total cell cycle length (T_C) and the duration of the cell cycle phases ($T_{S/G2/M/G1}$), see Box 1. Note that if determination of all cell cycle parameters is required, it is preferable to deduce them from a unique combined experiment on sibling tadpoles.

Labeling Tadpoles with EdU

1. Raise tadpoles in 0.1× MBS in Petri dishes in an incubator at 18°C–20°C until stage 41–42.
2. Transfer the tadpoles to 0.1× MBS containing 1 mM EdU.

If analysis is to be performed at embryonic stages (i.e., before or until stage 39/40), microinjection into the yolk is preferable to bathing, since it produces more homogeneous labeling. After microinjection, bathing in EdU solution is sufficient.

3. Incubate the tadpoles for the desired duration of EdU exposure.

Typical time periods are indicated in Figure 1A. For long exposure times, the medium should be renewed every other day so that EdU remains constantly available.

BOX 1. PRINCIPLES OF THE METHODS

Principle of T_C and T_S Determination through Cumulative EdU Labeling

Total cell cycle length (T_C) and S-phase length (T_S) can be determined using cumulative labeling with thymidine analogs such as bromo-2'-deoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU). Theoretically, this method presupposes that all cells studied are proliferating (or that nonproliferative cells can be distinguished and excluded) and uniformly distributed in the cell cycle, and that the population grows at a steady state (Nowakowski et al. 1989). The principle relies on the sequential administration of the chosen thymidine analog for a time that exceeds the presumed cell cycle length. The longer the pulse is, the more cells will go through S-phase and be labeled, until all proliferative cells are labeled (Fig. 1A,B). When determined for each pulse duration and plotted as a function of time, the proportion of labeled cells (also called the labeling index [LI]) increases linearly until a plateau is reached (Fig. 1C). The LI at the plateau allows estimation of the proportion of proliferative cells in the considered population (or growth fraction [GF]). As depicted in Figure 1A, the time needed to reach the plateau (T_{plateau}) is equivalent to $T_C - T_S$. A second equation derives from the fact that in a population of asynchronous cycling cells, the fraction of cells in a given phase is directly proportional to the length of that phase relative to total cell cycle length (Nowakowski et al. 1989). Thus, T_S/T_C can be deduced from the proportion of S-phase cells among proliferative cells at T_0 , which corresponds to the LI at T_0 (LI_0 ; extrapolated from the intercept of the best-fit line with the y-axis) corrected by the growth fraction. It is therefore equal to LI_0/GF . T_C and T_S can then be calculated from these two equations:

$$T_C - T_S = T_{\text{plateau}},$$

$$T_S/T_C = LI_0/GF.$$

Principle of T_{G2} Determination Using the PLM Method

T_{G2} determination relies on a paradigm called the percentage of labeled mitosis (PLM) (Quastler and Sherman 1959). Here again, the idea is to expose the tissue of interest to a thymidine analog, such as EdU, with increasing exposure times. One should then focus on the appearance of EdU-labeled cells among mitotic cells (immunostained with the late G2/M-phase marker phospho-Histone H3 or PH3 [Hendzel et al. 1997]). These represent the cells that were in S-phase and then went through G2 to finally reach M-phase during the EdU pulse (Fig. 1D,E). PLM increases sigmoidally as a function of time until all cells are double-labeled. Importantly, the duration of the EdU pulse required to reach the plateau is here much lower than in the cumulative labeling experiment. As depicted in Figure 1D, it grossly corresponds to $T_{G2} + T_M$. After plotting the results (Fig. 1F), the time required for half-maximal appearance of EdU labeling in the mitotic population is taken as an estimation of the average T_{G2} (Arai et al. 2011).

Principle of T_M Determination Using the Mitotic Index

M phase length determination relies again on the fact that the proportion of cells in a given phase is proportional to the ratio between the corresponding phase length and total cell cycle duration. Thus, with the mitotic index (MI) representing the percentage of mitotic cells in a given population, we can deduce the following equation:

$$T_M/T_C = MI/GF.$$

Evaluation of the mitotic index represents an easy way to qualitatively compare T_C in different experimental conditions under the hypothesis that M-phase duration is constant. If T_C and GF have been determined through EdU cumulative labeling, then T_M can be calculated.

FIGURE 1. Determination of T_C and T_S from a cumulative EdU labeling experiment and calculation of T_{G2} using the percentage of labeled mitosis (PLM) paradigm. (A) Outline of the experiment and schematic representation of cumulative labeling during cell cycle progression. Tadpoles are exposed to EdU for different durations and then fixed, sectioned and subjected to EdU labeling. Ten cells (numbered 1 to 10) are represented on the schematic. Cells become labeled (green) when they reach S-phase. Thus, the longer the EdU pulse is, the higher is the number of EdU-positive cells. As shown on the schematic, the time needed for all cells to be labeled corresponds to $T_C - T_S$ (follow, for instance, cell number 1). For simplification, the doubling of cells after mitosis is not represented. (B) Typical retinal section stained for EdU following a 4-h pulse at stage 41. Nuclei are counterstained with Hoechst. The panel on the right is a higher magnification of the dorsal CMZ. Red and white arrows point to EdU⁻ and EdU⁺ cells, respectively. Quantification can be performed in the stem cell compartment (delineated in orange and corresponding to the 3–5 most peripheral cells), in progenitors only, or in the whole CMZ (delineated in white using the plexiform layers as frontiers with the differentiated neural retina). (Legend continues on following page.)

Fixing, Dehydrating, and Sectioning Tadpoles

Here we describe microtome sectioning of tadpoles; alternatively, cryostat sectioning can be performed.

4. Anesthetize the tadpoles in 0.4 g/L MS222. Transfer them to glass vials using plastic pipettes.
5. Fix the tadpoles in 4% PFA with gentle agitation on a roller shaker for 2 h at room temperature or overnight at 4°C.
6. Wash the tadpoles three times for 5 min per wash in 1× PBS.
7. Dehydrate the tadpoles as follows.
 - i. Incubate three times for 30 min in 70% ethanol.
 - ii. Incubate once for 30 min in 95% ethanol.
 - iii. Incubate three times for 1 h in 100% ethanol.
Tadpoles can be stored in 100% ethanol at −20°C.
 - iv. Incubate overnight in 100% 1-butanol at room temperature.
8. Transfer the tadpoles to embedding molds. Remove the excess butanol and replace it with melted paraffin wax.
9. Incubate the tadpoles for ~6 h at 60°C. Change the paraffin wax 3–4 times during this incubation.
10. Align the tadpoles in each mold and let the paraffin solidify at room temperature.
11. Remove a paraffin block from the mold and cut around the tadpole with a razor blade. Place the embedded tadpole on a microtome (ventral side down, anterior in your direction). Trim at 50 μm to remove excess paraffin, and then section the tadpole at 10–12 μm.
12. Arrange successive ribbons containing eye sections on annotated slides covered with 3% glycerin albumen. Place the slides on a heating plate at 50°C. Wait for 20–30 sec and then remove the excess liquid. Allow the slides to dry for 10 sec on the heating plate.
13. Transfer the slides to a rack and incubate overnight at 37°C to dry.

After this step, slides can be stored at room temperature in a dry place.

FIGURE 1. (Continued) (CMZ) ciliary marginal zone, (GCL) ganglion cell layer, (INL/ONL) inner/outer nuclear layer, (IPL/OPL) inner/outer plexiform layers. (C) When labeling index (LI) is plotted as a function of time, a linear increase is observed until a plateau is reached. This allows determination of the proportion of cycling cells, or growth fraction (GF). The intercept of the linear part of the curve with the y-axis (LI_0) corresponds to $GF \times T_S/T_C$. The time taken for LI to reach the plateau (T_{plateau}) corresponds to $T_C - T_S$. The graph shown here is related to an EdU cumulative labeling experiment analyzed in the stem cell compartment and was adapted from Cabochette et al. (2015). (D) Outline of the experiment and schematic representation of PH3 and EdU labeling along with cell cycle progression. Tadpoles are exposed to EdU for different durations and then fixed, sectioned and subjected to double EdU/PH3 labeling. Ten cells (numbered 1 to 10) are represented on the schematic. During the EdU pulse, only cells that go through S-phase are EdU-labeled (green). This cohort then progresses through the cell cycle. If the duration of the EdU pulse is less than the G2-phase length, there will be no EdU-labeled cells among PH3-positive cells (red). When the duration increases, EdU-labeled cells appear among mitotic cells (yellow), as they had enough time to go through S and reach late G2/M phase. The minimal time required to obtain 100% double-labeled cells corresponds to the sum of G2- and M-phase lengths (follow, for instance, cell number 1). For simplification, the doubling of cells after mitosis is not represented. (E) Typical retinal sections (zoom on the dorsal CMZ; delineated in white) stained for both PH3 and EdU following a 2-h pulse at stage 41. Nuclei are counterstained with Hoechst. Red and yellow arrows point to $PH3^+ - EdU^-$ and $PH3^+ - EdU^+$ cells, respectively. Note that the shortness of the M-phase and thus the weak probability of finding mitotic cells in the stem cell compartment complicates the calculation of their G2-phase duration with this method. (F) Percentage of EdU-positive cells among mitotic cells plotted as a function of time. The time required for half-maximal appearance of EdU labeling in the mitotic population is taken as an estimation of the mean T_{G2} . The graph shown here is related to an experiment analyzed in the whole CMZ and was adapted from Cabochette et al. (2015).

Rehydrating Sections, Unmasking Epitopes, and Saturating Unspecific Fixation Sites

Citrate unmasking (Step 17) is necessary for PH3 detection on paraffin sections, but can be skipped if using cryostat sections or if performing EdU labeling only.

14. Transfer the slides to glass Coplin jars. Bathe twice for 10 min in 100% xylene to remove the paraffin wax.
15. Rehydrate the sections for 5 min per solution in 100%, 95%, 70%, and 50% ethanol.
16. Wash the slides three times for 5 min per wash in 1× PBS.
17. Microwave the slides in the unmasking citrate solution: Use maximal power (around 900 W) until boiling, and then use the defrost program (around 300 W) for 10 min. Cool the slides for 20 min at room temperature.
18. Wash the slides twice in dH₂O followed by three times in 1× PBS for 5 min per wash.
If using BrdU instead of EdU, add a denaturation step (45 min treatment in 2 N HCl followed by three washes in 1× PBS for 5 min per wash) before proceeding to permeabilization (Step 19).
19. Permeabilize the tissues for 20 min in PBT.
20. Transfer the slides to a horizontal surface in a humidified chamber. (Do not allow them to dry.) Add 800 µL per slide of blocking solution. Incubate for 20 min at room temperature.

Staining EdU-Labeled Cells

EdU staining relies on a copper-catalyzed covalent reaction between the fluorescent dye azide and the EdU alkyne (the "Click-iT" reaction). In contrast to BrdU detection, no DNA denaturation is required, since the small size of the dye azide allows it to easily gain access to DNA. Of note, EdU staining can be followed by immunofluorescence against a marker of interest in order to specifically label a given cell population. However, depending on the antibody used (e.g., anti-GFP[green fluorescent protein]), immunolabeling should be performed before the Click-iT reaction.

21. Discard the blocking solution from the slides and replace it with 200 µL per slide of the Click-iT reaction cocktail prepared according to the manufacturer's instructions.
22. Cover the slides with Parafilm or GelBond. Incubate for 30 min in a humidified chamber at room temperature in the dark.
23. Transfer the slides to glass Coplin jars and wash three times for 5 min per wash in PBT. (Allow the Parafilm or GelBond to detach by itself and then remove it.)

If performing both EdU and PH3 labeling, proceed to Step 24. If performing only EdU staining, proceed directly to Step 31.

Labeling PH3 and Counterstaining Nuclei

24. Repeat blocking as described in Step 20.
25. Discard the blocking solution from the slides and replace it with 200 µL per slide of primary antibody solution (anti-PH3 diluted 1:500 in blocking solution).
If using BrdU as a thymidine analog, perform a double immunostaining against both PH3 and BrdU.
26. Cover the slides with Parafilm or GelBond. Incubate in a humidified chamber overnight at 4°C or for 2 h at room temperature.
27. Transfer the slides to glass Coplin jars and wash three times for 5 min per wash in 1× PBS. (Allow the Parafilm or GelBond to detach by itself and then remove it.)
28. Transfer the slides to a horizontal surface and add 400 µL per slide of ALEXA-coupled secondary antibody (diluted 1:1000 in blocking solution).
29. Incubate the slides for 2 h in a humidified chamber at room temperature in the dark.
30. Transfer the slides to glass Coplin jars and wash three times for 5 min per wash in 1× PBS.

31. Incubate the slides for 10 min in Hoechst solution to counterstain the nuclei.
32. Wash the slides twice for 5 min per wash in 1× PBS.
33. Transfer the slides to a horizontal surface and apply a few drops of FluorSave reagent (or an equivalent antifade mounting medium) to each slide. Place coverslips carefully on top of the mounting medium.
34. Allow the slides to dry overnight at 4°C.
35. Proceed to imaging using a fluorescence microscope.

Counting Manually in the Ciliary Marginal Zone

Automatized counting using appropriate software such as FIJI is difficult in the ciliary marginal zone (CMZ). Because of the high nucleo-cytoplasmic ratio, nuclei appear packed together, rendering their automatic identification challenging. Manual counting must thus be performed, which probably represents the most arduous part of the described experiment. For each experimental condition, we recommend analyzing 6–10 sections (transverse sections that pass through the lens) per retina on 3–5 different retinas. This is usually enough for cell cycle kinetics experiments in the whole CMZ. If restricting the analysis to the stem cell compartment, anticipate needing a larger number of tadpoles to ensure a sufficient number of counted cells and reliable results.

36. Open the images using FIJI or Adobe Photoshop.
37. On each section, delineate the dorsal and ventral CMZ using the outer and inner plexiform layers as frontiers to separate CMZ cells from neuronal layers (Fig. 1B).
38. For all considered retinas, count on each CMZ section the number of PH3-positive cells, the number of EdU-labeled cells and the number of PH3-positive cells colabeled with EdU. Count the number of Hoechst-positive nuclei to estimate the total number of cells considered.
39. Report the results in an Excel spreadsheet. For each retina at each desired time point, calculate the percentage of (i) PH3-positive cells among total counted cells, (ii) EdU-labeled cells among total counted cells, and (iii) EdU-positive cells among total counted mitotic cells. Then, calculate mean percentages per retina and standard deviations for all these parameters.
40. If comparing different experimental conditions (e.g., gain or loss of function of your favorite gene vs. control), proceed to statistical analysis for each time point using the nonparametric Mann–Whitney test.

Calculating Cell Cycle Parameters

An explanation of the calculations is provided in Box 1.

Calculating T_C and T_S

41. Plot the average percentage of EdU-positive cells as a function of time (Fig. 1C).
42. Determine the growth fraction (GF) which corresponds to the percentage of labeled cells at the time at which the plateau is reached (T_{plateau}).
43. Determine the labeling index (LI_0) at T_0 by extrapolating the y intercept of the best-fit line.
44. Determine T_C and T_S using the following equations:

$$T_{\text{plateau}} = T_C - T_S,$$

$$LI_0 = GF \times (T_S/T_C).$$

Of note, more accurate calculations can be obtained using the spreadsheet developed by R. Nowakowski (Nowakowski et al. 1989), which allows drawing the best-fit line and calculates GF, T_C , and T_S using a nonlinear regression method. With his kind authorization, the spreadsheet is included with this protocol as a supplemental file (see Supplemental File S1). Report your results for each retina at each time point as

recommended and follow the included instructions. If publishing data gathered with this spreadsheet, Nowakowski et al. (1989) should be cited.

Calculating T_{G2}

45. Plot the average percentage of EdU-positive cells among mitotic cells as a function of time (Fig. 1F).
46. Determine the time at which 50% of cells are labeled.

This corresponds to an estimation of T_{G2} .

Calculating T_M and T_{G1}

47. Using the average percentage of PH3⁺ cells among total cells (MI) and the data gathered from EdU-cumulative labeling, calculate T_M using the following equation:
48. Deduce T_{G1} using the following equation:

$$T_M = (MI/GF) \times T_C,$$

$$T_{G1} = T_C - (T_S + T_{G2} + T_M).$$

DISCUSSION

During development, neural stem/progenitor cells undergo extensive proliferation to give rise to a nervous system with a defined size and precise cellular diversity. It is now well established that cell cycle progression is tightly linked to cell fate decisions (Ohnuma and Harris 2003; Molina and Pituello 2016). In the developing retina, cell cycle speed was shown to determine whether early or late neuronal cell types are produced (Decembrini et al. 2006, 2009). This led to the idea of a cellular clock dependent on cell cycle progression that would measure the length of the last cell cycle rather than time (Pitto and Cremisi 2010). In addition, cell cycle lengthening is known to causally contribute to the switch from proliferative to neurogenic divisions in the developing brain (Calegari et al. 2005; Lange et al. 2009; Salomoni and Calegari 2010). It has been proposed that neurogenic factor action may depend on whether G1 length provides enough time to do it (Calegari and Huttner 2003). However, G1 is not the only phase subjected to length variation. G2-phase duration was found to be important for the proper control of neuronal production in the developing spinal cord (Peco et al. 2012; Molina and Pituello 2016). Finally, S-phase length was also highlighted as a key feature distinguishing self-expanding stem/progenitor cells (long S) from progenitors committed to a neurogenic lineage (short S) (Arai et al. 2011; Cabochette et al. 2015; Turrero García et al. 2016). The mechanisms underlying such modulations of cell cycle kinetics remain quite elusive. Indeed, apart from cell cycle regulators such as cyclin and cyclin-dependent kinases, only a few factors and signaling pathways have been shown to regulate the cell cycle progression of neural precursors (Martynoga et al. 2005; Locker et al. 2006; Komada et al. 2008; Uribe and Gross 2010; El Yakoubi et al. 2012; Peco et al. 2012; Cabochette et al. 2015). The ability to measure the length of the different phases of the cell cycle in vivo following gene perturbation is thus of utmost importance to further understanding this process. The CMZ represents a powerful system for such studies since its location and spatial organization allows one to (i) easily delineate it from the nonproliferative neural retina, and (ii) topologically distinguish stem versus progenitor cells (Perron et al. 1998) and thus specifically measure their respective cell cycle kinetics.

Although the percentage of labeled mitosis (PLM) and EdU cumulative labeling represent efficient tools for in vivo examination of cell cycle kinetics, it is fundamental to keep in mind their intrinsic

limitations. First, and as mentioned in Box 1, EdU cumulative labeling and the PLM method presuppose that the studied proliferative population is in steady state. This criterion is barely fulfilled in developing tissues where total cell cycle duration and the lengths of the different phases vary dynamically throughout embryogenesis. It is, however, more or less met in the *Xenopus* CMZ, at least in late tadpole stages when retinal growth considerably slows down. A nonsteady state status is an important bias when calculating T_C , T_S , or T_{G2} because cells at the end of the labeling period might have a different cell cycle than those at its beginning. If significant variations in cell cycle speed are suspected during the labeling time period (for instance, based on the nonlinear aspect of the LI curve), an alternative method named “dual pulse labeling” can be considered. This technique is based on sequential administration of two different thymidine analogs such as EdU and IdU, CldU or BrdU (for the principle of this method, see Martynoga et al. 2005; for a protocol in *Xenopus*, see Auger et al. 2012). It allows estimating T_C and T_S using only one short time interval between injection of the two analogs (which diminishes bias from the nonsteady state of the considered population). A fundamental requirement for undertaking this experiment is to have antibodies able to discriminate between the two thymidine analogs in use.

A second and important drawback of PLM and EdU cumulative labeling (also true for dual pulse labeling) is that the calculated cell cycle parameters represent mean values which do not take into account the heterogeneity of the considered cell population. Live imaging of individual cells on neural tube slices from early chick embryos revealed striking heterogeneity, with T_C values ranging from ~9 to 28 h (Wilcock et al. 2007). The average value was found to be similar to that calculated from fixed tissue. This perfectly illustrates how analysis of populations may hide a high inter-individual variability of cycling behaviors. Although this has not been addressed yet, it is very probable that CMZ progenitors show such cell cycle heterogeneity. The same might be true for retinal stem cells as well. In addition, rare dormant cells have recently been described at the extreme tip of the zebrafish CMZ (Tang et al. 2017), suggesting that the *Xenopus* retinal stem cell cohort might be composed of both “activated” proliferating cells and “resting” quiescent ones, as shown in neurogenic zones of the adult mammalian or zebrafish brain (Than-Trong and Bally-Cuif 2015; Chaker et al. 2016). Finally, our personal observations suggest that the dorsal and ventral parts of the CMZ do not proliferate at the same rate, indicating an additional regional heterogeneity in cell cycle progression. Live imaging in the zebrafish CMZ has recently been established (Wan et al. 2016). In association with genetic tools allowing for visualization of cell cycle phases (such as the Fucci technology, Sakaue-Sawano et al. 2008), this may help in the close future to assess cell cycle kinetics at the single cell level and to estimate variability among stem/progenitor cells.

RECIPES

CaCl₂ (0.1 M)

11.1 g CaCl₂

Dissolve 11.1 g CaCl₂ in 1 L H₂O. Autoclave and store in aliquots at –20°C or 4°C.

MBS Salts (10×)

NaCl (880 mM)

KCl (10 mM)

MgSO₄ (10 mM)

HEPES (50 mM, pH 7.8)

Omit HEPES if MBS is to be used for oocyte maturation.

NaHCO₃ (25 mM)

Adjust pH to 7.8 with NaOH. Autoclave. Store at room temperature.

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Modified Barth's Saline (MBS)

CaCl₂ (0.1 M)

MBS salts (10×)

For a 1× solution of MBS, mix 100 mL of 10× MBS salts with 7 mL of 0.1 M CaCl₂. Adjust the volume up to 1 liter with H₂O. Store at room temperature.

ACKNOWLEDGMENTS

The methods described here have been developed and optimized by several past members of the laboratory. We thank in particular Warif El Yakoubi, Caroline Borday, and Pauline Cabochette. We are very grateful to R. Nowakowski for providing the Excel spreadsheet and for his authorization to make it accessible to whomever requests to use it. M.P.'s laboratory is supported by grants from the FRM, Association Retina France and Fondation Valentin Haüy.

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Cold Spring Harb Protoc; doi: 10.1101/pdb.prot105536 originally published online May 30, 2019

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