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***In vivo* assessment of neural precursor cell cycle kinetics in the amphibian retina**

Morgane Locker^{1*} and Muriel Perron^{1*}

¹ Paris-Saclay Institute of Neuroscience, CERTO-Retina France, CNRS, Univ Paris-Sud, University Paris-Saclay, 91405 Orsay, France.

* Corresponding authors: morgane.locker@u-psud.fr; muriel.perron@u-psud.fr

ABSTRACT

Cell cycle progression is intimately linked to cell fate commitment during development. In addition, adult stem cells exhibit specific proliferative behaviors compared to progenitors. Exploring cell cycle dynamics and its regulation is therefore a topic of utmost importance but constitutes a great challenge *in vivo*. We here 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 all along the animal life. The CMZ bears two tremendous advantages for cell cycle kinetics analyses. First, proliferative cells are sequestered in the CMZ, a region that can be delineated easily. 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 hereafter how to determine CMZ cell cycle parameters using a combination of (i) cumulative labelling assay, (ii) percentage of labelled mitosis (PLM) evaluation and (iii) mitotic index measurement. Taken together, these techniques allow 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 other developmental stages as well.

Short Title: *In vivo* assessment of retinal cell cycle kinetics

MATERIALS

Reagents

1-Butanol (100%)
Alexa-coupled secondary antibody (e.g. Thermo Fischer Scientific; 1 mg/mL)
Anti-PH3 antibody (e.g. Millipore 06-570; 1 mg/mL)
Blocking solution (PBT, 10% goat serum)
Click-iT EdU Alexa Fluor Imaging Kit (Thermo Fisher Scientific; follow the manufacturer's instructions for preparing stock solutions)
EdU (Thermo Fisher Scientific; 1 mM)
Ethanol (100%, 95%, 70%, 50%, dilutions in 1X PBS)
Antifade mounting medium like FluorSave reagent (e.g. Thermo Fischer Scientific)
Glycerin albumen (e.g. VWR; 3% freshly diluted in distilled H₂O from a 100% stock solution stored at -20°C)
Hoechst 33342 (5 μM)
Modified Barth's saline (MBS) stock solution (880 mM NaCl, 10 mM KCl, 10 mM MgSO₄, 50 mM HEPES, 25 mM NaHCO₃; pH 7.8)
Modified Barth's saline (MBS) 1X (10X dilution of MBS stock solution, 7 mM CaCl₂)
Paraffin wax (e.g. 00403 HISTOWAX®, Histolab)
Paraformaldehyde (PFA; 4%)
PBT (1X PBS, 0.2% Triton X-100)
Phosphate-buffered saline (1X PBS; pH 7.2-7.6)
Tricaine methane sulfonate 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 distilled water)
Xylene (100%)

Equipment

Disposable plastic embedding molds
Fluorescence microscope
Gel bond (Lonza) or parafilm
Glass Coplin jars
Heating plate at 50°C
Humid chamber
Incubator at 18-20°C, 37°C and 60°C
Microscope slides and coverslips
Microtome (e.g. Microm HM340E)
Petri dishes
Plastic pipettes and glass vials
Razor blades
Roller shaker

METHOD

Principle of the methods

1. **Principle of T_C and T_S determination through cumulative EdU labelling.** Total cell cycle length (T_C) and S-phase length (T_S) can be determined using cumulative labelling with thymidine analogues such as bromo-2'-deoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU). Theoretically, this method presupposes that all cells studied are proliferating (or that non-proliferative cells can be distinguished and excluded), 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 analogue 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 labelled, until all proliferative cells are labelled (**Figure 1A, B**). When determined for each pulse duration and plotted as a function of time, the proportion of labelled cells (also called labelling index or LI) increases linearly until a plateau is reached (**Figure 1C**). The LI at the plateau allows estimating 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 thus equal to LI_0/GF . T_C and T_S can then be calculated from these two equations:

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

$$T_S/T_C = LI_0/GF$$

2. **Principle of T_{G2} determination using the PLM method.** T_{G2} determination relies on a paradigm called the percentage of labelled mitosis (PLM) (Quastler and Sherman 1959). Here again, the idea is to expose the tissue of interest to a thymidine analogue, such as EdU, with increasing exposure times. What is followed in that case is the appearance of EdU labelled 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, then went through G2, to finally reach M-phase during the EdU pulse (**Figure 1D, E**). The percentage of labelled mitosis increases sigmoidally as a function of time until all cells are double-labelled. Importantly, the duration of the EdU pulse required to reach the plateau is here much lower than in the cumulative labelling experiment. As depicted in Figure 1D, it grossly corresponds to $T_{G2} + T_M$. After plotting the results (**Figure 1F**), the time required for half-maximal appearance of EdU labelling in the mitotic population is taken as an estimation of the average T_{G2} (Arai et al. 2011).
3. **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

MI (mitotic index) 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.

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.

Administration of EdU in tadpoles

4. Raise tadpoles in Petri dishes until stage 41-42 in 0.1X MBS in an incubator.
5. Transfer the tadpoles in 0.1X MBS containing 1 mM EdU. For long exposure times, the medium should be renewed every other day so that EdU remains constantly available. *If analysis is to be performed at embryonic stages (until stage 39/40), microinjection in the yolk is preferable since it gives more homogeneous labelling than bathing. Afterwards, bathing in EdU solution is efficient.*
6. Let the tadpoles in the incubator for the desired duration of EdU exposure (typical time periods are indicated in Figure 1).

Tadpole fixation, dehydration and sectioning

We here describe microtome sectioning but alternatively, cryostat sectioning can be performed.

7. Anesthetize tadpoles in 0.4 g/L MS222 and transfer them with plastic pipettes in glass vials.
8. Fix tadpoles in 4% PFA for 2 hrs at room temperature (RT) with gentle agitation on a roller shaker or overnight at 4°C.
9. Wash tadpoles 3X5 min in 1X PBS.
10. Dehydrate tadpoles: 3X30 min in 70% ethanol, 30 min in 95% ethanol, 3X1 hr in 100% ethanol (storage at -20°C is possible if needed), overnight in 100% 1-Butanol at room temperature.
11. Transfer the tadpoles in embedding molds, remove excess butanol and replace with melted paraffin wax.
12. Incubate at 60°C for ~6 hours. The paraffin wax should be changed 3-4 X during the day.
13. Align your tadpoles in each mold and let the paraffin solidify.

14. Remove the paraffin block from the mold, cut it around the tadpoles with a razor blade, place it on the microtome (ventral down, anterior in your direction) and section at 10-12 μM (you can start by trimming at 50 μM to get rid of the paraffin excess).
15. Arrange the successive ribbons containing eye sections on annotated slides covered with 3% glycerin albumen. Place the slides on the heating plate at 50°C. Wait 20-30 sec, remove excess liquid, dry 10 seconds on the heating plate and transfer at 37°C on a rack.
16. Let the sections dry overnight at 37°C (slides can afterwards be stored at RT in a dry place).

Rehydration, epitope unmasking and saturation of unspecific fixation sites

17. Place your slides in glass Coplin jars and bath 2X10 min in 100% xylene to remove the paraffin.
18. Rehydrate the sections in 100%, 95%, 70%, 50% ethanol (5 min each).
19. Wash 3X5 min in 1X PBS.
20. Microwave the slides in the unmasking citrate solution (maximal power - around 900 W - until boiling, then 10 min using the defrost program - around 300 W). Let it cool 20 min at RT and then wash 2X5 min in distilled H₂O and 3X5 min in 1X PBS.
Citrate unmasking is necessary for PH3 detection on paraffin sections but can be skipped if using cryostat sections or if performing EdU labelling only. In these cases, skip step 20 and proceed directly to step 21. If using BrdU instead of EdU, add a denaturation step (45 min treatment in 2N HCl, 3X5 min in 1X PBS) before permeabilization.
21. Permeabilize the tissues 20 min in PBT.
22. Place slides on a horizontal surface in a humidified chamber (don't let them dry).
23. Add 800 μL /slide of the blocking solution and incubate 20 min at RT.

EdU staining

EdU staining relies on a copper-catalysed covalent reaction between the fluorescent dye azide and the EdU alkyne ("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 the 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, immunolabelling should rather be performed before the Click-iT reaction (e.g. anti-GFP).

24. 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.
25. Cover with parafilm or gel bond and incubate 30 min in a humidified chamber and in darkness at RT.

26. Transfer the slides in glass Coplin jars and wash 3X5 min in PBT (let the parafilm or gel bond detach by itself and then remove it).
If performing both EdU and PH3 labelling proceed to step 27. If performing only EdU staining, proceed directly to step 34.

PH3 labelling and Hoechst staining

27. Proceed to a novel blocking 20 min at RT as before (steps 22-23).
28. Discard the blocking solution from the slides and replace it with 200 μ L per slide of primary antibody solution (anti-PH3 1:500 diluted in blocking solution).
If using BrdU as a thymidine analog, perform a double immunostaining against both PH3 and BrdU.
29. Cover with parafilm or gel bond and incubate overnight at 4°C (or alternatively 2 hrs at RT) in a humidified chamber.
30. Wash the slides 3X5 min in 1X PBS in glass Coplin jars (let the parafilm or gel bond detach by itself and then remove it).
31. Place slides on a horizontal surface and add 400 μ L per slide of ALEXA-coupled secondary antibody (1:1000 diluted in blocking solution).
32. Incubate 2 hrs in a humidified chamber and in darkness at RT.
33. Wash the slides 3X5 min in 1X PBS in glass Coplin jars.
34. Incubate 10 min in Hoechst solution to counterstain nuclei.
35. Wash 2X5 min in 1X PBS.
36. Place slides on a horizontal surface and apply few drops of the FluorSave reagent (or equivalent antifade mounting medium). Place coverslips carefully on top of the mounting medium.
37. Let the slides dry overnight at 4°C.
38. Proceed to imaging using a fluorescence microscope.

Manual counting in the ciliary marginal zone

Automatized counting using appropriate softwares such as FIJI is difficult in the CMZ. Indeed, due to 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 experiment. For each experimental condition, we recommend to analyze 6 to 10 sections/retina (transverse sections that pass through the lens) on 3 to 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 a larger number of tadpoles to ensure a sufficient number of counted cells and get reliable results.

39. Open the images using FIJI or Adobe Photoshop.
40. 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 (**Figure 1B**).
41. For all considered retinas, count on each CMZ section the number of PH3-positive cells, the number of EdU-labelled cells and the number of PH3-positive cells co-labelled with EdU. Count also the number of Hoechst-positive nuclei to estimate the total number of cells considered.
42. Report the results in an excel sheet and for each retina at each desired time point calculate the percentage of (i) PH3-positive cells among total counted cells, (ii) EdU-labelled 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.
43. If comparing different experimental conditions (e.g. gain or loss of function of your favorite gene *versus* control), proceed to statistical analysis for each time point using the nonparametric Mann-Whitney test.

Calculation of cell cycle parameters

44. Calculation of T_C and T_S
 - i. Plot the average percentage of EdU-positive cells as a function of time (**Figure 1C**).
 - ii. Determine your growth fraction, GF, which corresponds to the percentage of labelled cells at T_{plateau} (i.e. when the plateau is reached).
 - iii. Determine the labelling index LI_0 at T_0 by extrapolating the y intercept of the best-fit line.
 - iv. 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 Pr 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, it can be provided upon request. Report your results for each retina at each time point as recommended and follow the provided instructions. If publishing data gathered with this spreadsheet, (Nowakowski et al. 1989) should be cited.

45. Calculation of T_{G2}
 - i. Plot the average percentage of EdU-positive cells among mitotic cells as a function of time (**Figure 1F**).
 - ii. Determine the time at which 50% of cells are labelled which corresponds to an estimation of T_{G2} .
46. Calculation of T_M and T_{G1}

- i. Using the average percentage of PH3⁺ cells among total cells (MI) and the data gathered from EdU-cumulative labelling, calculate T_M as: $T_M = (MI/GF) \times T_C$
- ii. Deduce T_{G1} as: $T_{G1} = T_C - (T_S + T_{G2} + T_M)$.

DISCUSSION

During development, neural stem/progenitor cells undergo extensive proliferation to give rise to a nervous system with a defined size and a precise cellular diversity. It is now well established that cell cycle progression is tightly linked to cell fate decisions (Molina and Pituello 2016; Ohnuma and Harris 2003). 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 depending 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 important as well 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) to progenitors committed to a neurogenic lineage (short S) (Arai et al. 2011; Turrero García et al. 2016; Cabochette et al. 2015). The mechanisms underlying such cell cycle kinetics modulation 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 cell cycle progression of neural precursors (Locker et al. 2006; Martynoga et al. 2005; Komada et al. 2008; Uribe and Gross 2010; El Yakoubi et al. 2012; Cabochette et al. 2015; Peco et al. 2012). 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 dig further into this process. The CMZ represents a powerful system to do so since its location and spatial organization allows (i) to easily delineate it from the non-proliferative neural retina and (ii) to topologically distinguish stem *versus* progenitor cells (Perron et al. 1998) and thus to specifically measure their respective cell cycle kinetics.

Although, PLM and EdU cumulative labelling 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 above, EdU cumulative labelling 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 lengths of the different phases dynamically vary along embryogenesis. They are however more or less met in the *Xenopus* CMZ, at least for late tadpole stages, when retinal growth considerably slows down. A non-steady state status is an important bias when calculating T_C , T_S or T_{G2} because cells at the end of the labelling period might have a different cell cycle than those at its beginning. If important variations of cell cycle speed are suspected during the labelling time period (for instance based on the non-linear aspect of the LI curve), an alternative method named “dual pulse labelling” can be envisaged. This technique is based on the sequential administration of two different thymidine analogues such as EdU and IdU, CldU or BrdU (see Martynoga et al. 2005 for the principle and Auger et al. 2012 for a protocol in *Xenopus*). It allows estimating T_C and T_S using only one short time interval between injection of the two analogues (which diminishes the bias due to the non-steady state of the considered population). A fundamental requirement to undertake this experiment is to have antibodies able to discriminate the two used thymidine analogues.

A second and important drawback of PLM and EdU cumulative labelling (also true for dual pulse labelling) is that the calculated cell cycle parameters represent means 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 ranging from ~9 to 28 hours (Wilcock et al. 2007). The average was found similar to that calculated on fixed tissue. This perfectly illustrates how analysis on populations may hide a high inter-individual variability of cycling behaviours. Although this has not been addressed yet, it is very probable that CMZ progenitors exhibit 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 (Chaker et al. 2016; Than-Trong and Bally-Cuif 2015). Finally, our personal observations suggest that the dorsal and ventral parts of the CMZ do not proliferate at the same rate, suggesting an additional regional heterogeneity in cell cycle progression. Live imaging in the zebrafish CMZ has recently been set-up (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 close future to assess cell cycle kinetics at the single cell level and to estimate its variability among stem/progenitor cells.

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LEGEND

Figure 1: Determination of T_C and T_S from a cumulative EdU labelling experiment and calculation of T_{G2} using the percentage of labelled mitosis paradigm.

A. Outline of the experiment and schematic representation of cumulative labelling along with cell cycle progression. Tadpoles are exposed to EdU for different durations and then fixed, sectioned and subjected to EdU labelling. 10 cells numbered from 1 to 10 are represented on the schematic. They become labelled (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 labelled 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-hr 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 respectively point to EdU⁻ and EdU⁺ cells. 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). CMZ: ciliary marginal zone; GCL: ganglion cell layer; INL/ONL: inner/outer nuclear layer; IPL/OPL: inner/outer plexiform layers. **C.** When labeling indexes (LI) are plotted as a function of time, a linear increase is observed until a plateau is reached. This allows determining the proportion of cycling cells or GF. The intercept of the linear part of the curve with the y axis (LI_0) corresponds to $GF \cdot T_S / T_C$. The time for the LI to reach the plateau (T_{plateau}) corresponds to $T_C - T_S$. This graph is related to an EdU cumulative labelling 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 labelling along with cell cycle progression. Tadpoles are exposed to EdU for different durations and then fixed, sectioned and subjected to double EdU/PH3 labelling. 10 cells numbered from 1 to 10 are represented on the schematic. During the EdU pulse, only cells that go through S-phase are EdU-labelled (green). This cohort then progresses along the cell cycle. If duration of the EdU pulse is below G2-phase length, there will be no EdU-labelled cells among PH3-positive cells (red). When the duration increases, EdU-labelled 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% of double labelled 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-hr pulse at stage 41. Nuclei are counterstained with Hoechst. Red and yellow arrows respectively point to $PH3^+ - EdU^-$ and $PH3^+ - EdU^+$ cells. Note that the M-phase shortness and thus the weak probability of finding mitotic cells in the stem cells 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} . This graph is related to an experiment analyzed in the whole CMZ and was adapted from (Cabochette et al. 2015).

Figure 1

