Stem cell proliferative history in tissue revealed by temporal halogenated thymidine analog discrimination

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Detection of proliferating cells based on bromodeoxyuridine (BrdU) incorporation and determination of phenotype by immunofluorescence labeling are standard approaches for studying stem and progenitor cell populations in developing and adult tissue as well as in histopathology studies. We describe incorporation of different halogenated thymidine analogs for temporal discrimination of cell cycle in the rat. With equimolar delivery, these analogs are suitable for quantitative histological studies including assessment of the regulation of proliferation, clonal analysis and simultaneous profiling of cell phenotype relative to proliferative history.

Progression through cell cycle is a key event for the cell and for the tissue in which it resides. Progression through S phase, in which DNA replication occurs, is frequently examined as the hallmark event from which a cell can be 'birthdated'. Therefore, the labeled thymidine analogs [^{3}H]thymidine and BrdU can be used to identify proliferating cells[1]. By examining tissue for the location, type and number of labeled cells, it is possible to establish a chronology of cell proliferation and evaluate its contribution to tissue composition. Furthermore, by separating the administration of the labeled analogs at known intervals, it is possible to distinguish cells that reenter the cell cycle and continue to proliferate[2].

There are limitations on the use of [^{3}H]thymidine and BrdU for detecting the proliferative history of individual cells. Silver grains for [^{3}H]thymidine and reaction product for BrdU both appear in the nucleus, presenting difficulties with discrimination. Technical limitations hinder determination of spatial coexpression with additional markers of lineage commitment, so studies of cell proliferation and differentiation increasingly use multiple-immunofluorescence labeling to resolve the phenotypic identity of newly generated cells in developing and mature tissue[3–5]. As there are few reliable markers for adult stem cells resident in tissue, most studies take advantage of these cells' capacity for self-renewal through cell cycle reentry to label these cells (along with other, more committed progenitor cells) by systemic administration of BrdU. *In vitro* studies focused on temporal events in DNA replication use an alternative technique, replacing the bromo group with two other halogen moieties, chloro or iodo, with subsequent

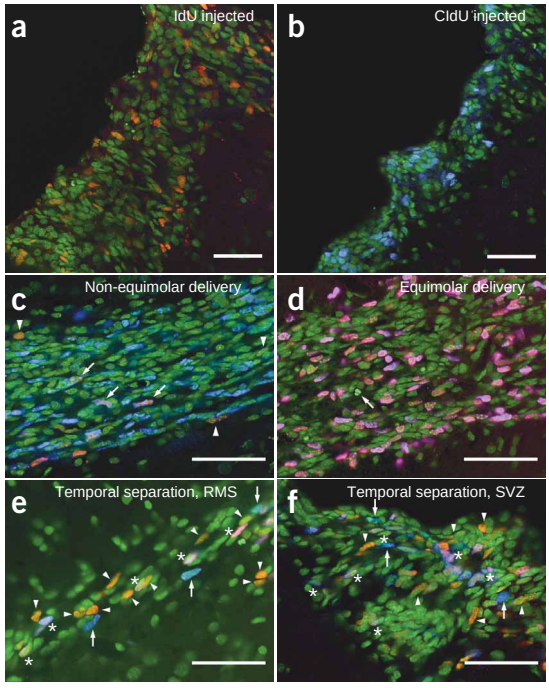

Figure 1 | Proliferative history of individual cells revealed by temporal discrimination of equimolar IdU and CldU delivery. (**a**–**f**) Cell cycle detection by pooled commercial antisera to BrdU in rat brain SVZ, one day following IdU or CldU delivery. IdU (**a**) was only detected by the Becton-Dickinson antibody (red), whereas CldU (**b**) was only detected by the Accurate antibody (blue). Sytox green counterstain shows location of all nuclei (**Supplementary Fig. 1** online). Simultaneous delivery of equivalent wt/vol analog dosage (**c**) demonstrated that cells in the RMS were not equally detected, with fewer IdU-positive cells (red, arrowheads) seen compared to CldU-positive cells (blue), and few cells showing coexpression of both analogs (purple; arrows). But, equimolar-concentration delivery (**d**) resulted in coexpression in all proliferating cells (purple). Incorporation was not detected in apoptotic profiles (arrow in **d**; also see **Supplementary Fig. 2** online). Separate equimolar delivery of IdU followed 24 h later by CldU permitted detection of cells reentering the cell cycle. Detection within the RMS (**e**) and SVZ (**f**) revealed three different proliferative histories– proliferation on day one (red only; arrowheads), day two (blue only; arrows), or both days (purple; asterisks). Also see **Supplementary Figure 3** online. Scale bars, 50 μm.

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discrimination by immunofluorescence microscopy[6–8]. To be useful in tissue, each thymidine analog must be clearly discriminated without detectable antibody cross-reactivity, it must label equivalent cell populations, and the staining procedures used must be compatible with additional fluorescence detection by lineage commitment markers. Finally, staining procedures must be suitable for three-dimensional detection of label colocalization for qualitative validity and for use with quantitative stereology. Here we report procedures using systemic administration of iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU) with specific detection by immunofluorescence that meet all of these criteria.

To evaluate the specific detection of thymidine analogs without cross-reactivity, rats were injected with BrdU, IdU or CldU, and regions of neurogenesis in adult brain (subventricular zone (SVZ), rostral migratory stream (RMS), and dentate gyrus) were examined for incorporation of the analogs (see the **Supplementary Methods** online for details). BrdU antisera generated in different species were tested simultaneously in brain sections from animals administered either IdU or CldU and some were found to be cross-reactive against these other halogenated thymidine analogs (**Fig. 1a**,**b**). Notably, two of these antisera were not equally cross-reactive and could be discriminated from each other (**Supplementary Fig. 1** online). As both antisera also recognized BrdU, it was not possible to coadminister BrdU with IdU and CldU to label a third cohort of proliferating cells.

Either thymidine analog should label cells undergoing S phase with equal probability, a critical requirement for both qualitative and quantitative studies of cell proliferation. But, we found that coadministration of equivalent wt/vol concentrations of IdU and CldU did not allow us to detect proliferating cells with equal probability (**Fig. 1c**). Because the molecular weights differed, this experiment was repeated with both analog concentrations adjusted to a common reference for molarity (**Supplementary Methods** online). Following simultaneous equimolar delivery of both IdU and CldU, all cells were colabeled with both markers (**Fig. 1d**). Thus, without equimolar administration, separate populations of proliferating cells can be distinguished, but the quantitative relationship between these populations cannot be established[9] (**Supplementary Fig. 2** online). To evaluate the proliferative history of individual cells, we administered equimolar concentrations of IdU and CldU 1 d apart to detect successive progression through S phase[10]. Immunofluorescence staining revealed three populations of proliferative cells in both the SVZ and RMS, reflecting DNA replication in the presence of only one or both analogs (**Fig. 1e**,**f** and **Supplementary Fig. 3** online). Thus, the proliferative history of individual cells can be quantitatively determined relative to the temporal interval of equimolar analog administration used in the experimental design.

To evaluate lineage commitment by a newly generated cell in the context of its proliferative history, animals received sequential equimolar administration of IdU and CldU. Many newly generated cells coexpressed early markers of neuronal migration and differentiation (**Fig. 2a–d**). Notably, our inability to detect the analogs in other cells that were positive for early neuronal markers suggests that this population of stem-progenitor cells is heterogeneous in regard to cell cycle. Markers for proliferation and for lineage commitment may not reside in the same cell compartment. Therefore, it is necessary to validate colocalization by acquiring a registered stack of sequential focal planes using confocal microscopy. A similar approach is required for quantifying cell populations using design-based stereology. Detection of equimolarly administered IdU and CldU by immunofluorescence labeling permits imaging throughout the cell distribution within the section thickness, making it suitable for validation of colocalization and for stereological quantitation[11,12] (**Fig. 2e**,**f**). Thus, we describe methods that overcome limitations of previous dual thymidine analog *in vivo* studies by (i) providing equimolar delivery, without which estimates of cell populations will produce distorted results[9] (**Fig. 1c**), and (ii) providing for simultaneous three-dimensional colocalization of multiple labels, without which phenotypic identity of new cells cannot be determined or counted with certainty[1,2,9] (**Fig. 2**).

The ability to detect dual cell cycle labels with markers of cell phenotype provides a powerful tool for (i) addressing the relationship between cell proliferative history and lineage determination, (ii) establishing clonal relationships between newly generated cells, and (iii) assessing proliferative status before and after experimental treatment in the same animal. Individual cell proliferative history may also make it possible to track the occurrence

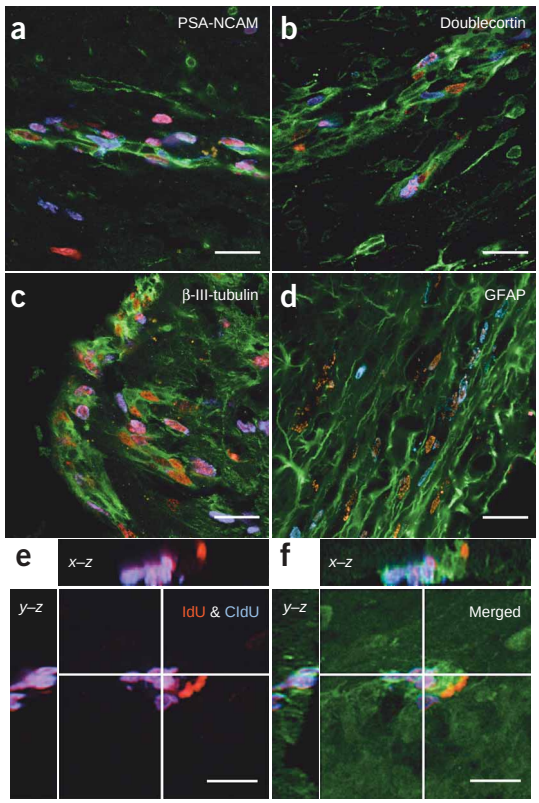

Figure 2 | Combined detection of cell proliferative history with lineage commitment. (**a–d**) Equimolar delivery of IdU (red) was followed 24 h later by CldU (blue) and the RMS was evaluated for coexpression of one or both (purple) analogs with early neuronal lineage markers (in green) PSA-NCAM (**a**), doublecortin (**b**) or β-III-tubulin (**c**), or the astrocyte marker GFAP (**d**). Not all analog-labeled cells coexpressed lineage commitment markers and not all committed cells underwent DNA replication during the interval examined. (**e**,**f**) To validate absolute coexpression of markers, sequential focal planes of high axial resolution were obtained and orthogonal images generated for a fixed position in space (crossing white lines) for colocalization (**e**) of IdU (red) and CldU (blue) combined with detection (**f**) of the lineage markers (here doublecortin, green). Scale bars, 20 μm.

of cell fusion by prelabeling grafted stem cells with one label and probing subsequently with the second label. This approach is equally suitable to the study of endogenous or grafted stem cells in other organ systems, such as muscle, gut, skin, and the hematopoietic system. The approach may also be useful for clinical studies of normal development, tissue repair, histopathology or cancer for which protocols for human administration have been approved[3].

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

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