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Analysis of Cell Proliferation and Homeostasis Using EdU Labeling

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Abstract

Determination of cellular proliferation and population turnover is an important tool for research on lymphoid cell function. Historically this has been done using radiolabeled nucleotides or nucleoside analogs, such as BrdU (5-bromo-2-deoxyuridine), that are incorporated into nascent DNA during S-phase. Recently, a new procedure was developed to label nascent DNA using EdU (5-Ethynyl-2-deoxyuridine). This new method overcomes limitations imposed by the procedure used to detect BrdU because EdU detection is based on an easily performed chemical reaction that does not require DNA denaturation, is quick and reproducible, and has a superior signal-to-noise ratio. This technique offers a wide range of opportunities to analyze cellular proliferation, population homeostasis, and cell marking procedures.

Keywords

EdU (5-ethynyl-2′-deoxyuridine); Click it chemistry; Proliferation; S-phase; DNA replication

1 Introduction

Measurement of the proliferative capacity and life span of defined cell types is fundamental for understanding population dynamics and homeostasis. One of the best approaches to accomplish this goal is to directly measure DNA synthesis. Early studies employed incorporation of radiolabeled thymidine followed by autoradiography and quantification in populations of cells in situ or isolated by a variety of different procedures [1]. However these procedures were laborious and it was difficult to analyze large numbers of cells. Later, methods using BrdU, a halogenated thymidine analog, were introduced to directly measure de novo DNA synthesis based on its incorporation during DNA synthesis and flow-cytometric detection using anti-BrdU antibodies were developed [2]. The great advance of this approach was that it utilized flow cytometry to simultaneously measure phenotypic markers, DNA content, and BrdU incorporation of a large number of individual cells. With some modifications, this overall approach has been used for the last 30 years to study proliferation dynamics and population homeostasis of defined cell subsets [1–3]. The method and timing of labeling depends on the experimental goals. For example, short term labeling identifies the proportion of cells actively replicating DNA during that period. When

this approach is coupled with DNA content analysis, it provides a rich source of information about cellular proliferation. For example, one can calculate the distribution of cells actively dividing according to their distribution in the G1-, S-, and G2/M-phases of the cell cycle. The number of resting cells can also be easily calculated. In addition, information about the duration of DNA synthesis, doubling time, and cell cycle dynamics is available. Due to the fact that EdU is not reutilized, “pulse-chase” studies can be done; dividing cells are labeled for a defined period followed by sampling and analysis at various timepoints. This technique is ideal for tracing step-wise cellular differentiation and population dynamics of post mitotic labeled cells during the chase period, and has been frequently used in studies on neural development and peripheral lymphocyte differentiation, some lasting almost 3 months [4]. Another application, using continuous labeling, allows calculation of proliferation rates of defined cell populations from the rate at which they incorporate labeled nucleotide analogs. The lifetime of any phenotypically defined lymphocyte population can be determined using continuous labeling experiments. The calculation is based on determining how long it takes for 100 % of the cells in a population to become labeled due to complete replacement of that population from precursor cells [4–6]. Specific applications of these procedures have been applied in a vast array of in vivo and in vitro experimental systems in biological models ranging from *C. elegans* and drosophila to humans [7].

In spite of the widespread use of BrdU to measure DNA synthesis, this procedure includes relatively harsh fixation procedures and requires opening of the DNA using heat, acid or DNase to expose the BrdU epitope and allow access for anti-BrdU antibodies. These factors limit phenotypic analysis using antibodies directed to specific proteins due to destruction of epitopes. Additionally, inconsistency in achieving exposure of the incorporated BrdU results in variable signal-to-noise ratios. Recently a new flow-cytometric method to label and detect nascent DNA using EdU has been developed and made commercially available by Life Technologies [8]. EdU, is a nucleoside analog of thymidine that is incorporated into DNA during S-phase just like BrdU and is not reactive in biological systems [9]. The EdU detection procedure technique uses a copper (I) catalyzed click reaction chemistry to covalently couple an azide modified fluorescent dye to incorporated EdU to form a stable triazole ring [8]. Because of the small size of the click detection reagents, no harsh DNA denaturation steps are required. As a result, EdU detection uses a simple protocol that takes less than 30 min with more reproducible results and greater signal-to-noise ratio. A variety of fluorochromes for EdU detection facilitate analysis of specific populations defined antibody based phenotypic profiling by specific proteins. It is likely that the advent of new and improved techniques using EdU to label nascent DNA will usher in a new wave of creative scientific exploration and exciting findings.

2 Materials

The components for EdU labeling and detection are included in kits purchased from Life Technologies that utilize various fluorochromes: Alexa Fluor 488, Alexa Fluor 647, or Pacifi c Blue.

2.1 In Vitro EdU Labeling

1. EdU (5-ethynyl-2′-deoxyuridine, Component A) (Life Technologies) (*see* Note 1).
2. Media appropriate for type of cell to be cultured.
3. Phosphate buffered saline (PBS).
4. DMSO.

2.2 In Vivo EdU Labeling

1. EdU (5-ethynyl-2′-deoxyuridine, Component A) (Life Technologies) (*see* Note 1).
2. Phosphate buffered saline (PBS).
3. Syringes with 25 G needles.
4. Sterile drinking water.

2.3 Stain Cell Surface Markers

1. FACS buffer (1× PBS, 0.5–1.0 % BSA, 0.1 % (W/V) sodium azide) (*see* Note 2).
2. Tubes compatible with flow cytometer to be used.
3. Fluorescently labeled antibodies.
4. Anti CD16 (2.4G2) antibody use at 5 μg/ml.

2.4 Fix and Permeabilize Cells

1. Click-iT fixative (Component D).
2. Click-iT saponin-based permeabilization and wash reagent (Component E).
3. De-ionized water.

2.5 EdU Detection

1. $CuSO_4$ (Component F).
2. Click-iT EdU buffer additive (Component G).

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1. After being dissolved, remaining EdU stock solution is stable for up to 1 year when stored at −20 °C. EdU solution should be portioned into single use aliquots to avoid freeze thawing. EdU is incorporated into DNA and is a potential mutagen. EdU has been identified as a teratogen in laboratory animals. Proper protective clothing should be used when handling EdU. Also proper procedures should be implemented to minimize contamination and dispose of waste according to institutional guidelines. Waste, including stock solutions, used media; animal cage litter, feces, urine, and water containing EdU should be considered as hazardous.
2. Fetal bovine serum can also be used at 3–10 % instead of BSA. Sodium azide is used as a preservative; its addition to the buffer is optional. Follow proper precautions when disposing of sodium azide to avoid accumulation of potentially explosive deposits in plumbing.

2.6 Optional Additional Antibody Staining for Intracellular Antigens or with RPE, PE-Tandem, or Qdot Antibody Conjugates

1. Cells that have been fixed, permeabilized, and stained for EdU with the Click-it reaction.
2. Fluorescently labeled antibodies.
3. 1× Click-iT saponin-based permeabilization and wash buffer.

2.7 Cell Cycle Stain

1. DAPI (4′,6-diamidino-2-phenylindole) solution 5 mg/ml in diH_2O.
2. FACS buffer.

2.8 Flow Cytometry Analysis

1. Flow cytometer and software to analyze data.

3 Methods

3.1 In Vitro EdU Labeling

1. Establish culture conditions where cells are proliferating.
2. Add 4 ml of DMSO or PBS to vial containing 10 mg EdU to make a 10 mM solution (*see* Note 1).
3. Add EdU to culture at 2–10 μM. EdU can be dissolved in DMSO or in aqueous solutions. EdU stock solution is 10 mM (*see* Note 3). Include cultures treated with vehicle alone for controls.
4. Culture for period to label nascent DNA (*see* Note 4). Harvest cells, count, and wash with PBS. Resuspend in FACS buffer at 1×10^7 cells/ml and keep on ice.

3.2 In Vivo EdU Labeling

1. Prepare EdU at 1 mg/ml in sterile phosphate buffered saline (PBS).
2. For short term or pulse labeling of adult mice, perform an intraperitoneal (IP) injection of 100–200 μl dissolved EdU (*see* Note 5). Harvest organs/cells at desired timepoints (*see* Note 6).

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3. Preliminary experiments should be done to determine optimal concentration for labeling and assess potential toxicity of DMSO or EdU on the cells of interest. In general 10 μM EdU has no detectable toxicity for a variety of different cell types. If toxicity is noted, reduction of EdU concentration or shortened labeling times may be indicated.
4. Preliminary experiments should be done to optimize labeling periods. In vitro EdU labeling can be detected in as little as 3 min. In most cases a 1–4 h labeling is sufficient. In general, labeling periods used for BrdU studies can be used as a good guideline for EdU labeling period, however EdU labeling can often be detected with shorter labeling periods than BrdU. Incorporated EdU is very stable and cells can be washed, fi xed and stored for months without loss of signal [8].
5. Alternatively mice can be injected with EdU twice 2–4 h apart to initiate the pulse labeling. Start timing the chase after the second injection. Newborn to 20-day-old mice can be injected with 50 μl 1 mg/ml EdU solution IP or SC.
6. Give enough time for EdU to diffuse and label proliferating cells. We have readily detected EdU incorporation in thymocytes after 60–90 min after injection.

3. For long term labeling, perform IP injection with 100 μl of 1 mg/ml EdU in PBS. Mice are then provided with drinking water ad libitum containing 0.3 mg/ml EdU (*see* Note 7). Replace drinking water with freshly prepared EdU every 2–3 days (*see* Notes 1 and 8).

4. Harvest lymphoid organs of interest and process into single cell suspensions. Count cells and wash with PBS. Resuspend in FACS buffer at 1×10^7 cells/ml and keep on ice.

5. If cells will not be stained with antibodies, place 100 μl of the cell suspension (1×10^6 cells) into FACS tubes, add 3 ml FACS buffer, pellet cells, remove supernatant and proceed to Subheading 3.4.

3.3 Stain Cell Surface Markers

1. Prepare a chart for the staining strategy listing fluorochromes, EdU detection and DNA stain. Antibodies using PE, PE-tandem, or Qdot(R) conjugates should not be used before the EdU detection step as their signal is reduced by the Click it reaction conditions. Currently, Life Technologies offers azides with Alexa Fluor 488, Alexa Fluor 647, and Pacific Blue. Also plan for additional samples for controls including unstained cells and single colors of each fluorochrome for compensation. When analyzing rare populations, it is useful to use additional control samples containing “all stains except one” to set the gates for the population identified by the missing fluorochromes.

2. Place 100 μl of the cell suspension (1×10^6 cells) into FACS tubes. Add 0.5 μg anti CD16/CD32 antibody in tubes to be stained with antibodies to block antibody binding by the Fc receptor and incubate on ice 10 min.

3. Set up single stain controls for each antibody. Reserve tubes with cells for EdU detection reagents and DNA stains to be added later. Incubate tubes on ice 15 min, add 3 ml FACS buffer, pellet cells and remove supernatant. Proceed to Subheading 3.4.

4. While control cells are staining, make a mixture with predetermined amounts of all desired antibodies and add an aliquot to samples to be stained. This procedure facilitates dispensing the antibodies and reduces tube-to-tube variability. Incubate tubes on ice 15–30 min, add 3 ml FACS buffer, pellet cells and remove supernatant. Proceed to Subheading 3.4.

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[7] EdU can also be administered subcutaneously (SC) every 3–4 h for up to 5 days if needed. Successful long term EdU labeling (in brain) has been achieved by injecting EdU every 3–4 h during a 12 h daytime period followed by a 12-h over-night period with no injections for a 5-day period [11]. Sequential injections are indicated for mice under 20 days old since they have not been weaned and do not drink much water. However the extra handling and disturbance may affect the experimental outcome so additional mice injected with vehicle alone on the same injection schedule should be included as controls. Long labeling periods may be toxic to some populations. Signs of distress, ruffled hair, lethargy and decreased thymus size compared to controls [12] suggest possible toxic effects. Labeling periods up to 5 weeks have been done with BrdU to study lymphocyte turnover. We have not observed toxicity with EdU labeling periods up to 7 days.

[8] Unlike BrdU, EdU is not light sensitive so water bottles do not need to be covered in foil.

3.4 Fix and Permeabilize Cells

1. All samples and controls should be fixed and permeabilized to ensure uniform characteristics when run on the flow cytometer. Prepare needed amount of 1× Click-iT saponin-based permeabilization and wash reagent by diluting the provided 10× stock (Component E) with water (*see* Note 9). A total of 3.6 ml per sample is needed for the standard reaction. If intracellular staining will be done, a total of 6.7 ml per sample is needed.
2. Gently drag tubes across a rack or use other method to break up the cell pellets from Subheadings 3.2, **step 5** and 3.3, **steps 3** and **4**.
3. Add 100 μl of Click-iT fixative (Component D) to each tube and mix well. Incubate the cells for 15 min at room temperature in the dark.
4. Add 3 ml of FACS buffer, pellet cells, and remove the supernatant (*see* Note 10).
5. Gently break up the cell pellet again and add 100 μl of 1× Click-iT saponin-based permeabilization and wash reagent. Mix well and proceed directly to Subheading 3.5 (*see* Note 11).

3.5 EdU Detection

1. Allow kit components to come to room temperature before opening.
2. Prepare a working solution of fluorescent azide for EdU detection by adding 130 μl of DMSO to Component B of the kit (Alexa Fluor 488, Alexa Fluor 647, or Pacific Blue) and mix well. Any remaining working solution will be stable for a year if stored at ≤−20 °C.
3. Add 2 ml of deionized water to the vial containing the Click-iT EdU buffer additive (Component G) to make a 10× stock solution and gently mix until fully dissolved. Any remaining stock solution should be dispensed into single use aliquots and is stable for a year when stored at ≤−20 °C.
4. Prepare a master mix of the components required for the Click-it reaction. For each tube mix together 438 μl PBS or TBS, 10 μl $CuSO_4$ (Component F), 2.5 μl fluorescent azide (prepared in Subheading 3.5, **step 1**), 50 μl 10× reaction buffer additive (prepared in Subheading 3.5, **step 2**). Scale up the mixture for the number of samples to be treated and add 500 μl to each tube. It is important to use the cocktail within 15 min of preparation. It is good practice to include a control sample of cells not exposed to EdU. In addition, these cells are needed for single staining compensation controls for intracellular antigens or antigens stained with RPE, PE-tandem, or Qdot antibody conjugates.
5. Incubate the mixture for 30 min at room temperature in the dark.

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9. The Click-iT permeabilization reagent maintains the morphological light scatter characteristics of leukocytes. It can be used with whole blood or cell suspensions containing red blood cells and will lyse red blood cells. The diluted 1× solution is stable for 6 months when stored at 2–6 °C while the 10× stock is stable for a year stored at −20 °C. In some cases, it may be more convenient to make up 500 ml 1× buffer. Note that this buffer contains sodium azide.
10. If red blood cell debris or hemoglobin is present in the sample, repeat the wash step before proceeding.
11. Cells can be held at this stage for up to 30 min if needed.

6. Add 3 ml of the Click-iT permeabilization and wash buffer (prepared in Subheading 3.4, **step 1**), pellet the cells and remove the supernatant.

7. Gently dislodge the cell pellet. If intracellular antibody staining is desired, add 100 μl Click-iT permeabilization and wash buffer and proceed to Subheading 3.6. Otherwise add 500 μl of the Click-iT permeabilization and wash buffer and go to Subheading 3.7 for DNA staining or Subheading 3.8 for flow cytometry.

3.6 Optional Staining for Intracellular Antigens or with PE, PE-Tandem, or Qdot Antibody Conjugates

1. Add predetermined amounts of antibodies to the cells and mix well. Incubate on ice for 30 min protected from light.

2. Add 3 ml of the Click-iT permeabilization and wash buffer, pellet the cells, and remove the supernatant.

3. Gently dislodge the cell pellet and add 500 μl of the Click-iT permeabilization and wash buffer and go to Subheading 3.7 for DNA staining.

3.7 Cell Cycle Stain

1. Dilute 5 mg/ml DAPI stock 1:100 with diH_2O and add 12.5 μl to each tube (3 μM final concentration) and incubate for 15 min at room temperature (*see* Note 12). Ensure that a tube of fixed and permeabilized cells is stained with only DAPI for compensation control. After incubation proceed to Subheading 3.8.

3.8 Flow Cytometry Analysis

1. Use unlabeled and single color controls to set up compensation on cytometer and run samples. Use “all but one” controls to set gates if needed (*see* Note 13).

2. Gate on the cell population of interest. Collect the fluorescent signal from DAPI or other DNA content dyes using linear amplification; all other fluorescent signals should be collected with logarithmic amplification. When measuring cellular DNA content on most flow cytometers, use a low flow rate (<500 events per second) during data collection. Use the same collection rate for all samples in the experiment (*see* Note 14).

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12. DAPI is a potential mutagen so use proper precautions when handling and disposing. DAPI stain may not compatible with Pacific Blue depending on whether a violet laser is used. Other DNA dyes include propidium iodide (FL2 channel) or 7-aad (7-Aminoactinomycin D) (FL3 channel). Life Technologies also sells several DNA dyes. RNase treatment is required if the dye binds RNA to ensure accurate DNA content profile and facilitate analysis.

13. Use this chart to determine the correct parameters to detect EdU with the different azides available from Life Technologies.

Azide	Excitation (mn)	Emission filter (or similar)
Alexa Fluor 488	488	Green (530/30 nm)
Alexa Fluor 647	633/635	Red (660/20 nm)
Pacific Blue	405	Violet (450/50 nm)

14. If additional dilution of cells is needed, ensure that wash buffer containing 3 μM DAPI is used to prevent reduction of fluorescent signal.

3. Cell clumping is often observed when processing samples for flow cytometry. When analyzing DNA content, it is important to distinguish between G1 doublets from a G2-M single event. To discriminate the doublet from the singlet, plot the width (*W*) versus area (*A*) for the channel used in a dot plot graph. A population of single cells will form a diagonal. *W* increases with the diameter of the clump, while the *A* of a G1 doublet and the G2/M single cell is the same. Therefore, discrimination of G1 doublets and clumped cells from G2/M single cells can be made by gating out events that deviate with greater width from the diagonal as shown in Fig. 1a [10].

4. Using the desired phenotypic gate, the percent cells that synthesized DNA and incorporated EdU during the pulse period can be determined with a simple histogram (Fig. 1b). Plotting EdU versus DAPI provides more information about the cell cycle (Fig. 1c). In this example, human epithelial cells were pulsed with EdU for 2 h and stained with 7-aad and Pacifi c Blue azide. The bivariate analysis shows that 59 % of these cells synthesized DNA during the pulse and represent cells in S-phase (Fig. 1c). Twenty percent of the cells were quiescent and remained in G1 phase while 13 % did not synthesize DNA and were in G2-M phase (Fig. 1c). A population of stable quiescent cells can be identified by lack of EdU incorporation over a longer labeling period, while a population of cycling cells will become uniformly labeled.

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References

1. Darzynkiewicz Z, Traganos F, Zhao H, Halicka HD, Li J (2011) Cytometry of DNA replication and RNA synthesis: Historical perspective and recent advances based on “click chemistry”. Cytometry A 79:328–337 [PubMed: 21425239]
2. Dolbeare F, Gratzner H, Pallavicini MG, Gray JW (1983) Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. Proc Natl Acad Sci USA 80: 5573–5577 [PubMed: 6577444]
3. Gray JW, Dolbeare F, Pallavicini MG, Beisker W, Waldman F (1986) Cell cycle analysis using flow cytometry. Int J Radiat Biol Relat Stud Phys Chem Med 49:237–255 [PubMed: 3510993]
4. Tough DF, Sprent J (1994) Turnover of naiveand memory-phenotype T cells. J Exp Med 179:1127–1135 [PubMed: 8145034]
5. Parretta E, Cassese G, Santoni A, Guardiola J, Vecchio A, Di Rosa F (2008) Kinetics of in vivo proliferation and death of memory and naive CD8 T cells: parameter estimation based on 5-bromo-2’-deoxyuridine incorporation in spleen, lymph nodes, and bone marrow. J Immunol 180:7230–7239. [PubMed: 18490722]
6. Rocha B, Penit C, Baron C, Vasseur F, Dautigny N, Freitas AA (1990) Accumulation of bromodeoxyuridine-labeled cells in central and peripheral lymphoid organs: minimal estimates of production and turnover rates of mature lymphocytes. Eur J Immunol 20:1697–1708 [PubMed: 2209684]
7. Cavanagh BL, Walker T, Norazit A, Meedeniya AC (2011) Thymidine analogues for tracking DNA synthesis. Molecules 16:7980–7993 [PubMed: 21921870]

8. Salic A, Mitchison TJ (2008) A chemical method for fast and sensitive detection of DNA synthesis in vivo. Proc Natl Acad Sci USA 105:2415–2420 [PubMed: 18272492]
9. Buck SB, Bradford J, Gee KR, Agnew BJ, Clarke ST, Salic A (2008) Detection of S-phase cell cycle progression using 5-ethynyl-2'-deoxyuridine incorporation with click chemistry, an alternative to using 5-bromo-2'-deoxyuridine antibodies. Biotechniques 44: 927–929 [PubMed: 18533904]
10. Nunez R (2001) DNA measurement and cell cycle analysis by flow cytometry. Curr Issues Mol Biol 3:67–70 [PubMed: 11488413]
11. Clarke LE, Young KM, Hamilton NB, Li H, Richardson WD, Attwell D (2012) Properties and fate of oligodendrocyte progenitor cells in the corpus callosum, motor cortex, and piriform cortex of the mouse. J Neurosci 32: 8173–8185 [PubMed: 22699898]
12. Tough DF, Sprent J, Stephens GL (2007) Measurement of T and B cell turnover with bromodeoxyuridine. Curr Protoc Immunol Chapter 4:Unit 4 7

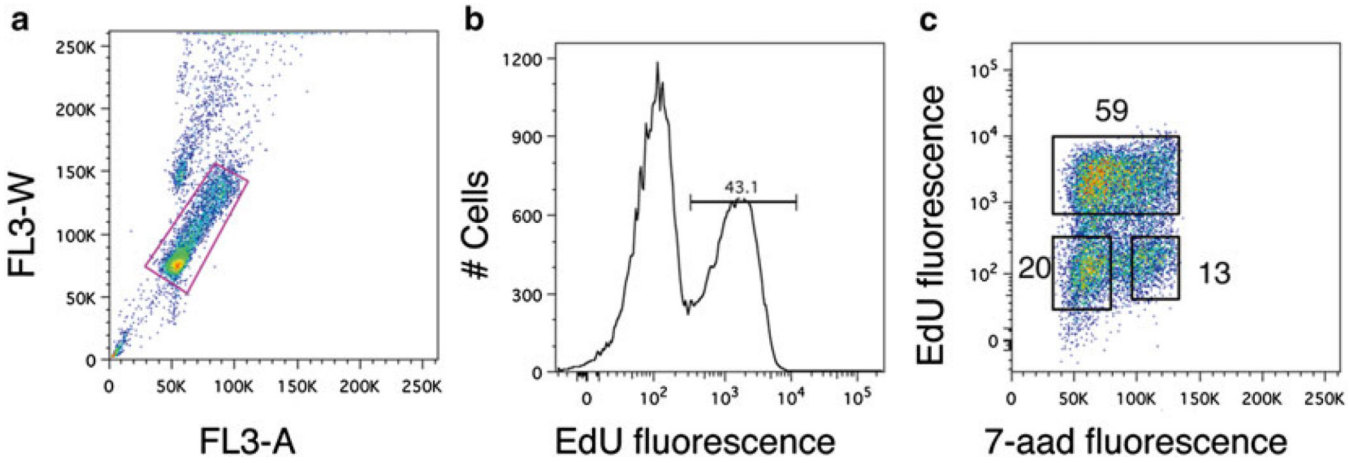

Fig. 1.

Human epithelial cells were cultured with EdU for 2 h then stained with Pacific Blue azide and 7-aad. (**a**) 7-aad fluorescence was collected using the FL3 channel and plotted area (FL3-A) versus width (FL3-W) to allow discrimination between clumped doublets and single cells shown within the *rectangular gate*. (**b**) Cells were gated based on size and the percentage of EdU labeled cells is shown as a histogram. (**c**) Bivariate analysis of EdU versus 7-aad staining is shown for cells gated as in (**a**). *Numbers* shown denote percentages. Clockwise from the *top* are gates that show cells in S-phase (59 %), G2/M (13 %) and G0/G1 (20 %)