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Measuring Proliferation of Vascular Smooth Muscle Cells Using Click Chemistry

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Corresponding Author:	Lucile Wrenshall Wright State University Dayton, OH UNITED STATES
Corresponding Author's Institution:	Wright State University
Corresponding Author E-Mail:	lucile.wrenshall@wright.edu
Order of Authors:	Victoria Wong
	Prakash Arumugam
	Lucile Wrenshall
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TITLE:

Measuring Proliferation of Vascular Smooth Muscle Cells Using Click Chemistry

AUTHORS AND AFFILIATIONS:

Victoria Wong[1], Prakash Arumugam[1], Lucile E. Wrenshall[1,2]

[1]Department of Neuroscience, Cell Biology, and Physiology, Wright State University, Dayton, OH, USA

[2]Department of Surgery, Boonshoft School of Medicine, Wright State University, Dayton, OH, USA

Corresponding author:
Lucile E. Wrenshall (lucile.wrenshall@wright.edu)

Email addresses of co-authors:
Victoria Wong (victoria.wong@wright.edu)
Prakash Arumugam (prakash.arumugam@wright.edu)

KEYWORDS:

click chemistry, proliferation, vascular smooth muscle cells, adherent cells, fluorescence, EdU

SUMMARY:

Proliferation is a critical part of cellular function, and a common readout used to assess potential toxicity of new drugs. Measuring proliferation is, therefore, a frequently used assay in cell biology. Here we present a simple, versatile method of measuring proliferation that can be used in adherent and non-adherent cells.

ABSTRACT:

The ability of a cell to proliferate is integral to the normal function of most cells, and dysregulation of proliferation is at the heart of many disease processes. For these reasons, measuring proliferation is a common tool used to assess cell function. Cell proliferation can be measured simply by counting; however, this is an indirect means of measuring proliferation. One common means of directly detecting cells preparing to divide is by incorporation of labeled nucleoside analogs. These include the radioactive nucleoside analog ^{3}H-thymidine plus non-radioactive nucleoside analogs such as 5-bromo-2' deoxyuridine (BrdU) and 5-ethynyl-2'-deoxyuridine (EdU). Incorporation of EdU is detected by click chemistry, which has several advantages when compared to BrdU. In this report, we provide a protocol for measuring proliferation by the incorporation of EdU. This protocol includes options for various readouts, along with the advantages and disadvantages of each. We also discuss places where the protocol can be optimized or altered to meet the specific needs of the experiment planned. Finally, we touch on the ways that this basic protocol can be modified for measuring other cell metabolites.

INTRODUCTION:

Proliferation is a critical part of cellular function[1,2]. Control of proliferation influences normal processes such as development, and pathologic processes such as cancer and cardiovascular

disease. Hyperplastic growth of vascular smooth muscle cells, for example, is thought to be a precursor to atherosclerosis[3]. Changes in cell proliferation are also used to assess potential toxicity of new drugs. Given its widespread impact, measuring proliferation is a mainstay of many cell biology-based laboratories.

Cell proliferation can be measured by simply counting cells if the cell population of interest divides fairly rapidly. For slower growing cells, cell counts may be less sensitive. Proliferation is often measured by incorporation of labeled nucleoside analogs. Although the gold standard is ^{3}H-thymidine incorporation, many laboratories are getting away from this method given the availability of newer, non-radioactive alternatives. These include cytoplasmic fluorescent dyes, detection of cell cycle associated proteins, and incorporation of non-radioactive nucleoside analogs such as 5-bromo-2' deoxyuridine (BrdU) and 5-ethynyl-2'-deoxyuridine (EdU)[4].

Cytoplasmic dyes, such as carboxyfluorescein diacetate succinimidyl ester (CFSE), detect proliferation because the intensity of the dye halves each time the cell divides[5]. This technique is commonly used in flow cytometry for non-adherent cells. It has not been used much for adherent cells, but with the new generation of imaging plate readers this may change. Detection of cell cycle associated proteins through antibody-based techniques (flow cytometry, immunohistochemistry, etc.) is often used for tissues or non-adherent cells. These cells/tissues must be fixed and permeabilized prior to staining. Nucleoside analogs BrdU and EdU are similar in approach to ^{3}H-thymidine, but without the inconvenience of radioactivity. Incorporation of BrdU is detected by anti-BrdU antibodies, and therefore cells must be fixed and permeabilized prior to staining. In addition, cells must be treated with DNase to expose the BrdU epitope.

Incorporation of EdU is detected by click chemistry, in which alkyne and azide groups "click" together in the presence of catalytic copper. Either group can serve as the biosynthetically incorporated molecule or detection molecule[4]. Commercially available nucleoside analogs have the alkyne group attached. For proliferation assays, therefore, the alkyne functionalized nucleoside analog is detected by an azide conjugated to a fluorescent dye or other marker. Incorporation of EdU has several advantages over BrdU. First, because alkyne and azide groups are not found in mammalian cells, this interaction is highly specific with a low background[6]. Because both groups are small, cells do not need to undergo DNA denaturation to expose the nucleoside analog as required for BrdU[7]. Finally, click chemistry is highly versatile, and can be used for the metabolic labeling of DNA, RNA, protein, fatty acids, and carbohydrates[8,9,10,11]. If the metabolically labeled target of interest is on the cell surface, live cells can be labeled[12]. In addition, cells can be further processed for immunofluorescent staining with antibodies.

The following protocol describes the use of EdU incorporation and click chemistry to measure proliferation in human vascular smooth muscle cells (**Figure 1**). We show three different ways incorporation of EdU can be measured, representative results from each, and their advantages and disadvantages. Measuring proliferation via click chemistry is particularly useful for adherent, slower growing cells. In addition, the morphology of the cell is well maintained and antibody-based staining can also be performed.

PROTOCOL:

1. Detection of EdU using fluorescent label

1.1. Stock solutions

1.1.1. Prepare a 5 mM EdU stock solution by adding 12.5 mg of EdU to 10 mL of double distilled H_2O.

1.1.2. Prepare the labeling solution components: 200 mM tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) (100 mg in 1.15 mL H_2O), 100 mM $CuSO_4$ (15.95 mg in 1 mL H_2O; make fresh), 10 mM Cy3 picolyl-azide (1 mg in 95.3 mL H_2O, stored in 5 µL aliquots at -20 °C), and 1 M sodium ascorbate (200 mg in 1 mL H_2O; make fresh).

1.1.3. Prepare resazurin stock solution at a concentration of 0.15 mg/mL. Filter sterilize and store 1 mL aliquots at 4 °C.

NOTE: Picolyl azide is available conjugated to several different fluors, biotin, and horseradish peroxidase (HRP).

1.2. Label vascular smooth muscle cells (VSMC) with EdU.

NOTE: Human VSMCs were isolated from iliac arteries via outgrowth from explanted pieces of tissue. When grown in serum free media with insulin, transferrin, selenium these cells express VSMC markers smoothelin, smooth muscle myosin heavy chain (SM-MHC) and SMC αactin[13].

1.2.1. Plate vascular smooth muscle cells at 2 x 10^4/mL in Dulbecco's modified Eagle's medium (DMEM) in a 96 well plate.

NOTE: Cells are grown at 37 °C in smooth muscle cell media with 2% fetal bovine serum.

1.2.2. (Optional) Allow several hours to overnight for adherence. Add 20 µL resazurin stock to each well. Incubate at 37 °C for 3 h. Read the fluorescence signal in a plate reader (560_{ex}, 594_{em}). Replace media with fresh DMEM.

NOTE: This step is optional. Resazurin is used to normalize cell numbers and account for well-to-well variability in plating[14].

1.2.3. Add phosphate buffered saline (PBS) or platelet derived growth factor (PDGF) 30 ng/mL to 3−6 wells per treatment at the beginning of the culture period and incubate for 72 h.

1.2.4. Dilute 5 mM EdU stock to 1 mM. Add 2 µL to each well (final concentration 20 µM) for the last 24 h of the 72 h culture period. Do not add EdU to one set of replicates. These wells will be used to determine background fluorescence/luminescence.

NOTE: The duration of culture and labeling with EdU is optimized for smooth muscle cells but may need to be modified per cell type.

1.3. Fix cells in paraformaldehyde (PFA).

1.3.1. Remove media and add 150 µL of 4% PFA (in PBS) per well for 10 min at room temperature.

1.3.2. Remove PFA and add 150 µL of 1% polyethylene glycol *tert*-octylphenyl ether (TX-100) (in water) per well for 30 min at room temperature.

1.3.3. Remove TX-100 by washing three times with PBS.

CAUTION: PFA is toxic, handle with care.

NOTE: The protocol can be paused here. Fixed cells can be stored in PBS at 4 °C.

1.4. Detect incorporated EdU.

1.4.1. Make labeling solution (10 mL per 96 well plate, using inner 60 wells) just prior to use by adding reagents from stock solutions in the following order: THPTA 20 µL, $CuSO_4$ 20 µL, Cy3 picolyl azide 5 µL, Na ascorbate 100 µL to PBS for a total volume of 10 mL.

1.4.2. Remove PBS from wells and add 150 µL of labeling solution to each well. Incubate 30 min at 37 °C. To detect all nuclei, add 4',6-diamidino-2-phenylindole (DAPI) to each well. Read on a fluorescence plate reader (Cy3 550_{ex}, 570_{em}; DAPI 350_{ex}, 470_{em}) or image on a microscope.

NOTE: The protocol can be paused here. Fixed and stained cells can be stored in PBS at 4 °C and imaged at a later time.

2. Detection of EdU using luminescence

2.1. Complete sections 1.1–1.3.

2.2. Prepare Tris-buffered saline with 0.1% polysorbate 20 (TBST).

2.3. Block wells.

2.3.1. Add 150 µL of blocking buffer (**Table of Materials**) to each well and incubate for 90 min at room temperature.

2.3.2. Remove blocking buffer and wash three times with 200 µL of PBS.

2.4. Detect incorporated EdU.

2.4.1. Make labeling solution as directed in step 1.4.1, substituting biotin picolyl azide for Cy3 picolyl azide.

2.4.2. Wash wells five times with 200 µL of TBST, with shaking, 3 min per wash.

2.4.3. Quench endogenous peroxidases with 150 µL of 0.3% H_2O_2 for 20 min at room temperature.

2.4.4. Remove H_2O_2 and wash five times with 200 µL of TBST, with shaking 3 min per wash.

2.4.5. Dilute streptavidin-horseradish peroxidase (SA-HRP; 1:200) in TBST and add 50 µL to each well. Incubate 1 h at room temperature.

2.4.6. Wash five times with 200 µL of TBST, with shaking, 3 min per wash.

2.4.7. Add 50 µL of chemiluminescent enzyme-linked immunosorbent assay (ELISA) substrate (**Table of Materials**) to each well.

2.4.8. Read immediately in plate reader capable of detecting luminescence.

REPRESENTATIVE RESULTS:

In **Figure 2**, we demonstrate the outcomes of three different experiments measuring proliferation of VSMC in response to PDGF. After growing the cells in media formulated for SMCs, we carried out the experiment in serum free DMEM, to eliminate any potential effects of serum or growth factors on proliferation. In **Figure 2A** we compare results, from the same experiment, using a fluorescent vs luminescent readout. To read these plates, we used a multimode microplate reader that can read absorbance, fluorescence, and luminescence (see **Table of Materials**).

Using the plate reader in scanning well mode, fluorescent readings are averaged over the entire well. This mode alleviates potential inaccuracies due to differences between the center and edges of the well. However, if very few nuclei are positive, reading results in this way likely underestimates the “number” of positive cells when only a few are positive. Number, with this readout, means fluorescence relative to that of another well, and not exact cell numbers. In addition, if there is a fiber or a clump of dead cells that picks up fluorescence, this will be included in the area scan. However, the advantage of using the plate reader is time. While reading in scanning mode takes 15−20 seconds per well, it is automated as opposed to the personal time-intensive method of taking pictures and analyzing them using imaging software.

To correct potential underestimates from the above method, we converted the fluorescent scan to a homogenous, liquid readout in which cells taking up EdU are detected with an azide-biotin moiety, which is in turn detected with streptavidin-horseradish peroxidase (see **Figure 1**). The amount of horseradish peroxidase is then quantified using a standard substrate formatted for

ELISA. The advantage of this technique is a more homogenous readout, and **Figure 2A,B** shows examples of experiments where this method was employed. In addition, the plate can be read in seconds. There are disadvantages to this method, however. First, the background with this method is higher than with fluorescence. To decrease the background, we incorporated a blocking step and multiple washes. As a reflection of this background, negative controls (untreated cells) tend to be higher, and the fold increase in proliferation is lower. Second, as with the fluorescence readout, any fibers or dead cells that pick up the detection reagents will add to the total luminescence.

In an attempt to decrease variability in the above experiments, we normalized our results to initial cell counts. For proliferation assays, normalization has to be done on live cells at the beginning of the assay before the cells divide. In addition, the assay cannot affect cell function or incorporation of EdU. Given these constraints, we chose to use metabolism as a reflection of cell number via resazurin[14]. Results with and without normalization are shown in **Figure 2B**. However, in a separate set of experiments we found that this method is not sensitive enough to detect small differences in cell numbers. In addition, any error in this readout introduces more error in the entire experiment. For these reasons, we have elected to take extraordinary care to plate cells as evenly as possible, and not perform a normalization step.

To be sure we were getting the most accurate results possible, we reverted to fluorescent staining (section 1) and counting cells using an inverted fluorescence microscope. The advantage of this method is that one can physically see and count the positive cells, ensuring the most accuracy (**Figure 3**). Any fluorescent debris can be excluded from the count. Background counts without EdU are 0 as there is no visible fluorescence, and therefore no background to subtract. The main disadvantage of this method is time. To count nuclei in cells plated in a 96 well plate, we take 3 pictures per well of both EdU positive cells and total cells (DAPI), requiring 6 images per well. We take pictures vertically from the midsection of the top to the bottom of the well, taking care not to overlap and count cells twice. Counting total cells gives us a second piece of data that confirms our proliferation results. However, in light of the relatively slow doubling time of VSMC (36−48 h), the fold increase in total cells stimulated with PDGF vs controls is much lower than the fold increase of EdU positive cells, which is why we measure incorporation of EdU (**Figure 2C**).

The most efficient and accurate way to count EdU positive and total cells is by using an imaging plate reader. This method combines the accuracy of counting with the speed of automation. The main drawback is that this piece of equipment is not available at every institution. In comparing automated vs manual counts, the EdU positive manual count was essentially identical to the automated count (**Figure 2C**, left), as were the total unstimulated counts (**Figure 2C**, right). However, the total manual cell count was lower in the PDGF-stimulated cells. Because the cells tend to grow in the center of the well, with smaller numbers the automated and manual counts were nearly identical. However, with high numbers of cells we missed more cells at the edges, and manual counting was less accurate.

FIGURE LEGENDS:

Figure 1: Overview of EdU detection by fluorescence or luminescence. VSMC or other adherent cells are treated under conditions of interest. EdU is added for the last 24 h of the culture period and incorporated into the DNA of dividing cells. Incorporated EdU is then detected using fluorescence (protocol section 1) or luminescence (protocol section 2).

Figure 2: Measuring proliferation with different readouts using incorporation of EdU and click chemistry. (**A**) VSMCs were cultured in the presence or absence of PDGF. EdU was added the last 24 h of culture, and incorporation was detected by either fluorescence (protocol section 1) or luminescence (protocol section 2). (**B**) VSMCs were treated as described in panel A, and incorporation of EdU was measured by luminescence. Results were normalized to initial cell counts using resazurin viability assay. (**C**) VSMCs were treated as in panel A, and incorporation of EdU was measured by fluorescence. EdU positive cells were counted automatically, using an imaging plate reader, or manually by imaging and then counting using imaging software. Results shown are the average ± standard deviation of 3–6 replicates per treatment.

Figure 3: Visualization of EdU positive cells following click fluorescence protocol. VSMC were treated as described in **Figure 2**, and incorporated EdU was detected using fluorescence (protocol section 1). Each image is a single well of a 96 well plate. Total nuclei are shown in blue (DAPI) and EdU positive nuclei are green. Scale bar = 50 μm.

DISCUSSION:

Incorporation of EdU is a simple, straightforward way to measure cell proliferation; it is particularly useful for adherent cells[13]. Our protocol uses smooth muscle cells, but it is applicable to any adherent cell (epithelial, endothelial, etc.). Although the protocol is not complicated, the one critical step is making the labeling solution — the ingredients must be added in the order listed. In addition, like chemiluminescent Western blots, if using the luminescence readout the plate must be read immediately.

Several parts of the protocol can be modified or optimized as needed. One part is the duration of incubation with EdU. Because our goal was to detect as many proliferating cells as possible, we added EdU for a full 24 h prior to fixing and staining. However, this timing likely measures cells in both S and G2/M phases. To label only cells in S phase, Cecchini et al. advise that even slowly dividing cells be incubated with EdU for no longer than 6 hours[7]. Another part of the protocol that can be optimized is the amount of chelator. Alkyne-azide reactions require a copper catalyst ($CuSO_4$). THPTA is a copper chelator that maintains copper in the necessary oxidation state. The ratio of chelator to copper can be varied, to increase alkyne-azide binding. We observe bright staining with a chelator:copper ratio of 2:1, in the context of also using picolyl azide. The picolyl moiety adds chelating activity which decreases the concentration of copper needed and increases the efficiency of the reaction[15]. Chelator:copper ratios can be increased, usually up to 5:1, to optimize per investigator needs. A better chelator, 2-[4-({bis[(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl]amino}methyl)-1H-1,2,3-triazol-1-yl]acetic acid (BTTAA), is now commercially available[16]. This chelator was not commercially available when we first derived this protocol. Finally, although we use paraformaldehyde as a fixative in this protocol, cells can be fixed with

methanol, which also obviates the permeabilization step. Fixing with methanol can be helpful if one wants to combine immunofluorescent staining of specific cellular proteins with incorporation of EdU.

One drawback of this method is the potential cytotoxicity of EdU, particularly when continuously labeling with EdU for days as opposed to a pulse label of hours. Depending on the cells used, EdU has been found to cause cell cycle arrest or even apoptosis[17]. This toxicity is thought to be due to defective DNA replication from the addition of artificial nucleosides[18]. Studies have shown this toxicity to be cell line dependent and concentration dependent[17]. We did not observe any problems with toxicity in our studies.

In summary, measuring proliferation via EdU and click chemistry has several advantages over other commonly used methods such as BrdU or ^{3}H-thymidine incorporation. These include avoiding radioactivity, high specificity with low background, and no need for harsh denaturation steps. Once established, click chemistry is highly versatile, and can be easily modified for the metabolic labeling of RNA, protein, fatty acids, and carbohydrates.

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DISCLOSURES:
The authors have nothing to disclose.

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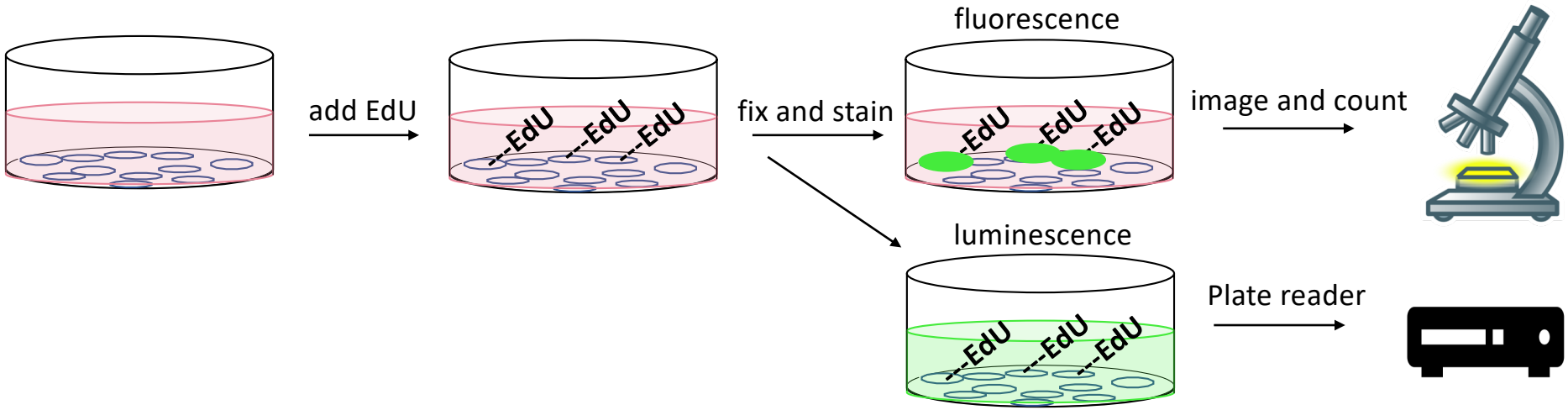
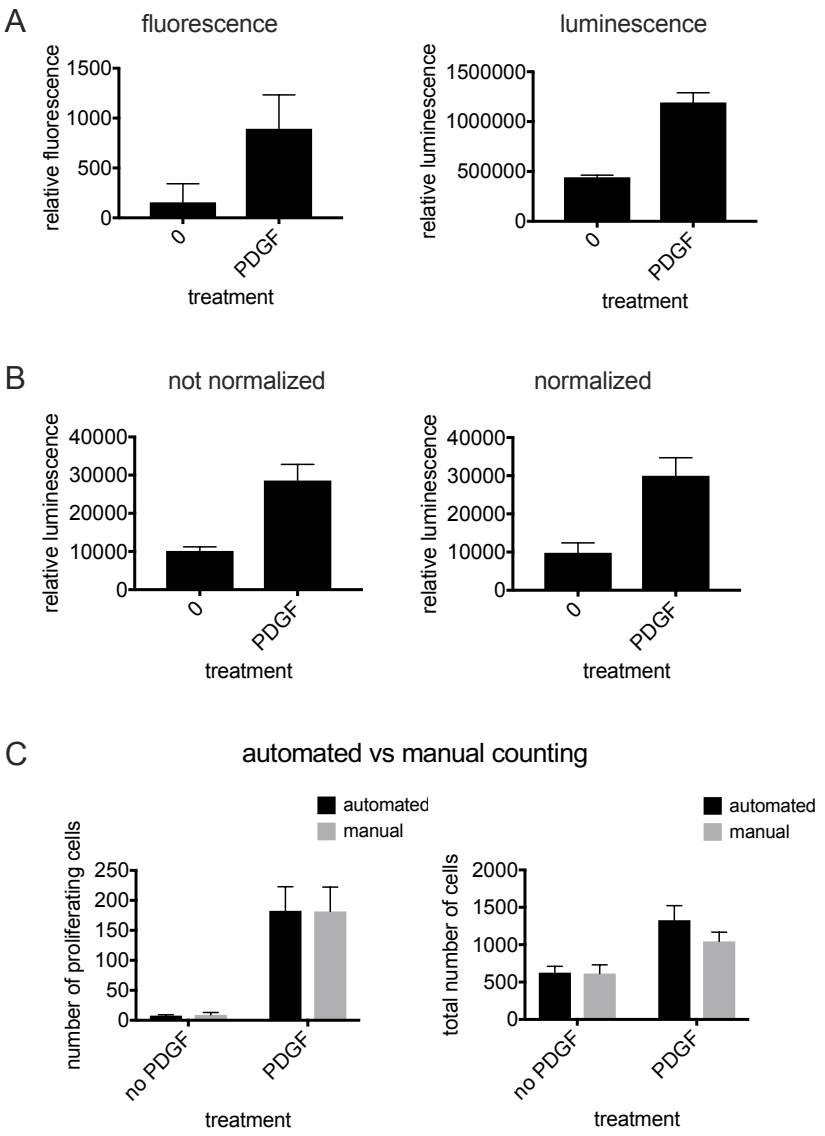
Figure





Figure

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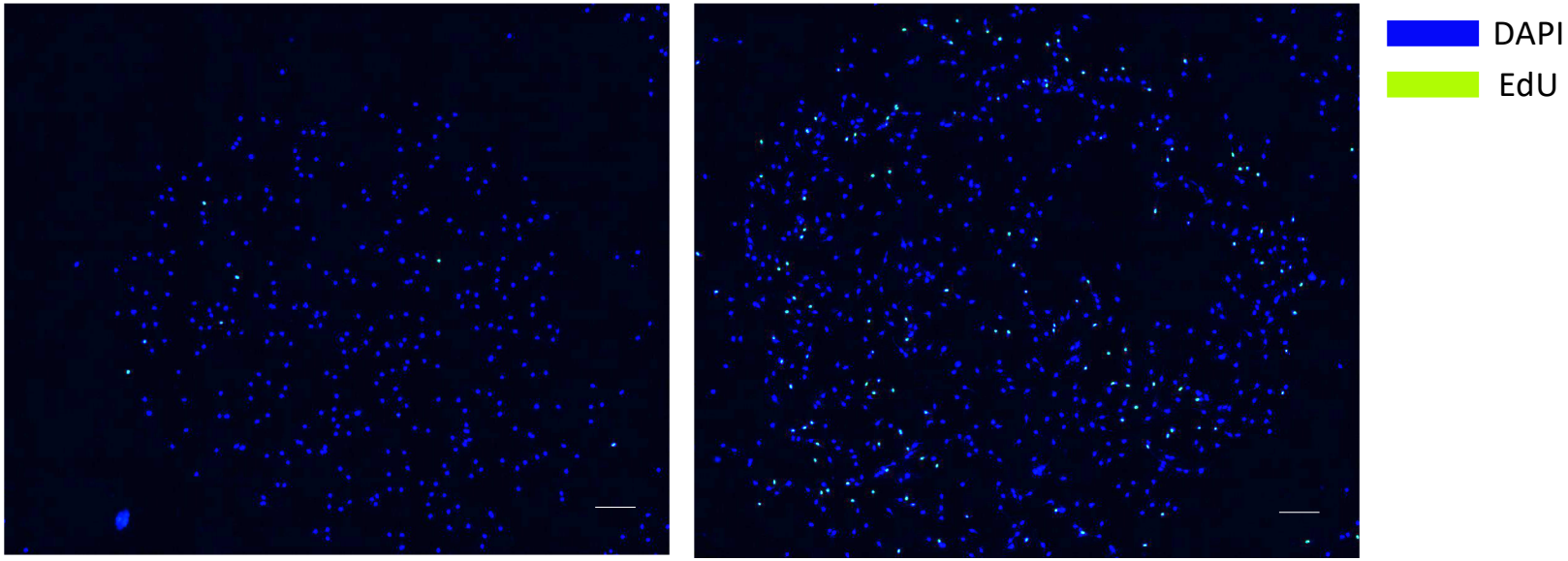

no PDGF

PDGF

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
5-Ethynyl-2'-deoxyuridine	Carbosynth	NE08701	
biotin picolyl azide	Click Chemistry Tools	1167-5	
CuSO4	Fisher Scientific	C1297-100G	
Cy3 picolyl azide	Click Chemistry Tools	1178-1	
Cytation Plate Reader	Biotek		
EVOS Microscope	Thermo Fisher Scientific		
Hydrogen peroxide solution	Sigma-Aldrich	216763	
Na ascorbate	Sigma-Aldrich	11140-250G	
NucBlue Fixed Cell Stain Ready Probes	Invitrogen	R37606	DAPI nuclear stain
Odyssey Blocking Buffer	Li-Cor	927-40000	blocking buffer
Paraformaldehyde	Electron Microscopy Services	15710	
PBS	Fisher Scientific	SH3002802	
Sodium Chloride	Sigma Life Science	S3014-5kg	
Streptavidin Horseradish Peroxidase Conjugat	Life Technologies	S911	
Super Signal ELISA Femto	Thermo Scientific	PI137075	ELISA substrate
Synergy Plate Reader	Biotek		
THPTA	Click Chemistry Tools	1010-100	
Tris Hydroxymethyl Aminomethane Hydrochloride (Tris-HCl)	Fisher Scientific	BP153-500	
Triton X 100	Bio-Rad	1610407	
Tween 20	Fisher Scientific	BP337-100	

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1 Alewife Center #200
Cambridge, MA 02140
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CORRESPONDING AUTHOR

Name:	Lucile Wrenshall
Department:	Neuroscience, Cell Biology, and Physiology; Surgery
Institution:	Wright State University
Title:	Professor
Signature:	Lucile Wrenshall
Date:	2/28/19

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Dear JoVE reviewers,

Thank you for the opportunity to revise our manuscript " Measuring Proliferation of Vascular Smooth Muscle Cells Using Click Chemistry". We appreciate the suggestions of the reviewers and believe their feedback has significantly improved the quality of the manuscript. Please find attached a point-by-point response to reviewer's concerns. Our responses to the reviewers' comments are in italics.
We hope that you find our responses satisfactory and that the manuscript is now acceptable for publication.

Sincerely,

Lucile E. Wrenshall, M.D., Ph.D., FACS
Professor of Surgery and Neuroscience, Cell Biology, and Physiology
Wright State University

Reviewer 1 Major Concerns:

1. The first two paragraphs have no citations. Please include citations for historic/data-evident statements.
References have been added to the first 2 paragraphs.

2. Where were the vascular smooth muscle cells derived from (what species)? Include source (vendor, cat#), also state if they were a cell line or generated from other technology.
We use human vascular smooth muscle cells (VSMC) isolated from iliac arteries using an explant method. This information has been added to the protocol as a note under item 2.1, "Label vascular smooth muscle cells with EdU".

3. Were the VSMCs differentiated and characterized? What kind of VMSCs were used here? Was there any form of injury or intoxication besides PDGF? if yes, the responses could be altered.
The smooth muscle cells are propagated in media using serum and are therefore by definition proliferative or synthetic rather than differentiated or contractile. No other treatment was added prior to PDGF. The VSMC are characterized by the smooth muscle cell markers smooth muscle cell alpha actin and smoothelin. This information has been added to the text.

4. Have you performed experiments with shear stress in the 2 D/cell culture model?

We have not performed these experiments. In addition, because we are using VSMC as a means to demonstrate the use of click chemistry to measure proliferation in adherent cells we are focusing on this assay.

5.What particular disease indication are you working on? Details of that are also missing. Don't need elaborate details but it will justify use of this methodology for categorized disease functional areas.
Proliferation of VSMC contributes to intimal hyperplasia and atherosclerosis. This indication has been added to the introduction.

6. Section 2.2 Cite literature.
I am assuming that the reviewer wants the resazurin to be cited. A reference was added to the note below section 2.1 and to the results section where resazurin is mentioned.

7. Section 3-provide excitation emission wavelengths for fluorescence read outs.
Cy3 excitation and emission wavelengths have been added to item 4.1 number 2 in the protocol.

Minor Concerns:
8. Include photographs of fluorescence imaging. It is good to see quantified data but pictures of the staining also have value. Can be an added as a separate figure or together with quantified data.
An image of EdU positive cells has been added (new Figure 3).

9. Since this is a methods journal, it is extremely helpful if a flow-chart or pictorial guide is provided for the steps. Do this for all the proposed protocols.
A pictorial guide has been added (new Figure 1).

10. A little more detail on click chemistry can be provided.
More detail on click chemistry has been added to the introduction.

Reviewer #2:
Manuscript Summary:
The topic is very interesting and novel. The paper is well organized in general.

Minor Concerns:
As the author mentioned: "...we touch on the ways that this basic protocol can be modified for measuring other cell metabolites.", we hope to see more details in this aspect, especially the application in other types of cells.
This information has been added to the beginning of the discussion.