Video Article

Detecting Glycogen in Peripheral Blood Mononuclear Cells with Periodic Acid Schiff Staining

Mahdieh Tabatabaei Shafiei[1], Catalina M. Carvajal Gonczi[1], Mohammed Samiur Rahman[2], Ashley East[3], Jonathan François[3], Peter J. Darlington[1,3]

[1]Department of Biology, Centre for Structural and Functional Genomics, PERFORM Centre, Concordia University

[2]Department of Chemistry and Biochemistry, Centre for Structural and Functional Genomics, PERFORM Centre, Concordia University

[3]Department of Exercise Science, Centre for Structural and Functional Genomics, PERFORM Centre, Concordia University

Correspondence to: Peter J. Darlington at peter.darlington@concordia.ca

URL: https://www.jove.com/video/52199
DOI: doi:10.3791/52199

Keywords: Immunology, Issue 94, Periodic acid Schiff, glycogen, soleus muscle, lymphocyte, peripheral blood mononuclear cells, metabolism, immunology, amylase

Date Published: 12/23/2014

Citation: Tabatabaei Shafiei, M., Carvajal Gonczi, C.M., Rahman, M.S., East, A., François, J., Darlington, P.J. Detecting Glycogen in Peripheral Blood Mononuclear Cells with Periodic Acid Schiff Staining. *J. Vis. Exp.* (94), e52199, doi:10.3791/52199 (2014).

Abstract

Periodic acid Schiff (PAS) staining is an immunohistochemical technique used on muscle biopsies and as a diagnostic tool for blood samples. Polysaccharides such as glycogen, glycoproteins, and glycolipids stain bright magenta making it easy to enumerate positive and negative cells within the tissue. In muscle cells PAS staining is used to determine the glycogen content in different types of muscle cells, while in blood cell samples PAS staining has been explored as a diagnostic tool for a variety of conditions. Blood contains a proportion of white blood cells that belong to the immune system. The notion that cells of the immune system possess glycogen and use it as an energy source has not been widely explored. Here, we describe an adapted version of the PAS staining protocol that can be applied on peripheral blood mononuclear immune cells from human venous blood. Small cells with PAS-positive granules and larger cells with diffuse PAS staining were observed. Treatment of samples with amylase abrogates these patterns confirming the specificity of the stain. An alternate technique based on enzymatic digestion confirmed the presence and amount of glycogen in the samples. This protocol is useful for hematologists or immunologists studying polysaccharide content in blood-derived lymphocytes.

Video Link

The video component of this article can be found at https://www.jove.com/video/52199/

Introduction

Periodic acid Schiff (PAS) staining is an immunohistochemical technique that is widely used in muscle research and diagnostics. It is also utilized as a diagnostic tool on blood samples. The technique works by applying periodic acid solution to the sample, which oxidizes units within the polysaccharide creating aldehyde groups that react with the colourless Schiff's reagent thereby producing a deep magenta product. The steps of this procedure are shown in **Figure 1**. The stain turns anything with polysaccharides magenta, including glycogen, glycoproteins, glycolipids, mucins, or other molecules with polysaccharide moieties.

PAS staining is often used to measure glycogen levels in muscle fibers. Muscles tissue sections are ideal for the technique as they firmly attach to the slide and withstand multiple washing and staining steps. Glycogen is most present in fast twitch Type II muscle fibers, which have a high demand for rapid ATP production requiring glycogen for maximum performance[1,2]. Glycogen is a branched polymer of glucose that can be broken into free glucose through the action of glycogen phosphorylase enzymes. In times of rest and nutritional-sufficiency, glycogen is replenished through the process of glycogenesis, while in times of nutritional insufficiency or high-energy demand; glycogen is broken down into glucose by glycogenolysis. From as early as the 1950's clinician scientists have explored PAS staining on blood samples to analyze glycogen content in various diseases[3-7]. For example, in Pompe disease-a bonafide glycogen storage disease- white blood cells accumulate large amounts of glycogen that differs significantly from healthy controls[8].

This video-article demonstrates an adapted version of PAS staining for use on peripheral blood mononuclear cells (PBMC) samples from venous blood of healthy human subjects. PBMCs contain mostly lymphocytes of the T lymphocyte and B lymphocyte families, as well as other immune cells such as natural killer cells and monocytes. The first purification step removes erythrocytes, neutrophils, and other granulocytes. This technique provides data on a concentrated proportion of lymphocytes allowing for more robust enumeration of PAS-positive cells as compared to using whole blood smears.

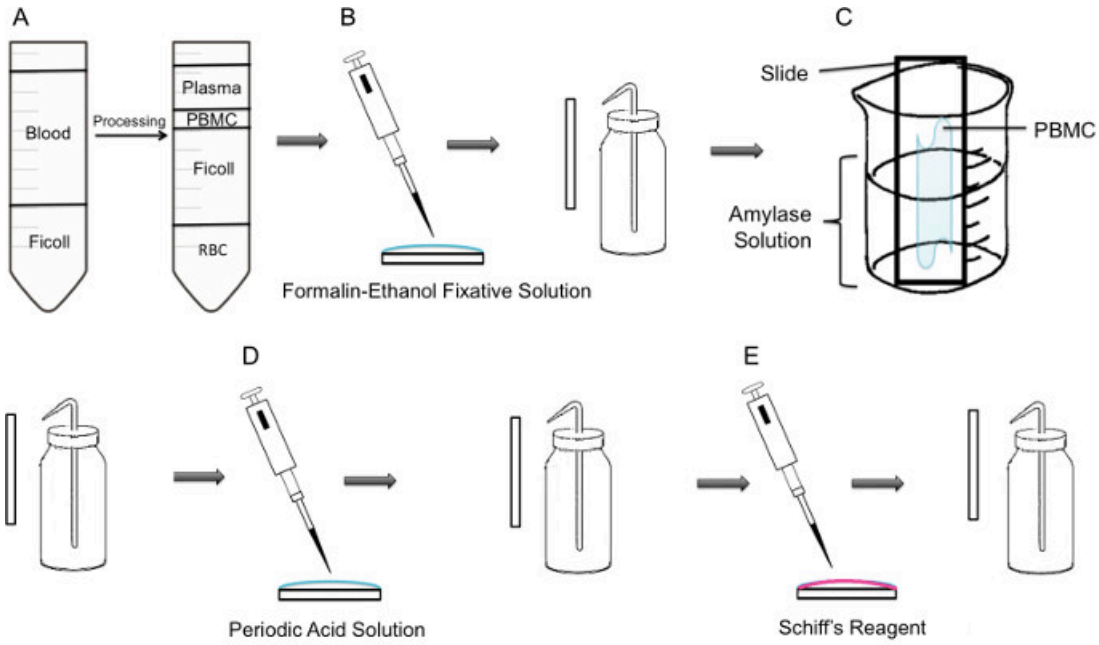

Figure 1: Step by step methodology of PAS staining on PBMC. (A) First, isolation of PBMC is achieved through ficoll gradient, the left panel shows the preparation before centrifugation, the right panel shows it after centrifugation where the buffy coat containing the PBMC is observed in the center of the tube. **(B)** Isolated PBMCs are fixed onto the slide using formalin-ethanol fixative solution. The slide is gently rinsed with distilled water from a plastic wash bottle. **(C)** The slide is then placed in a 100 ml beaker half way filled with amylase solution, which will dissolve glycogen. The slide is gently rinsed. **(D)** The slide is treated with periodic acid solution, where oxidation of saccharides takes place. Slides are gently rinsed; this will remove the excess periodic acid and stop the oxidation step. **(E)** When the Schiff reagent is added to the slides, it will react with aldehydes created during the oxidation step. This colorless reagent will then result in a deep red magenta product. Slides are gently rinsed to remove the excess Schiff reagent.

Protocol

Research with human blood samples was approved by Concordia University Ethics Review Board, certificate number 10000618. The work on mouse muscle was approved by Concordia University Ethics Review Board, certificate number 2010BERG.

1. PBMC Isolation from Whole Blood

NOTE: Carry out this procedure in a biosafety cabinet using sterile technique and manufacturer-sterilized equipment.

1. Carefully pour 10-15 ml of whole blood from the heparinized (anti-coagulant) blood collection tubes into a 50 ml sterile conical tube. For minor blood spills, wipe with ddH_2O and 70% EtOH using cleaning tissue.
2. Dilute the blood in the conical tube to a 1:1 ratio with Phosphate Buffer Saline (PBS 1x) pH 7.4. Ensure that the maximum total volume does not exceed 30 ml.
3. Mix gently using a serological pipette gun avoiding bubbles.
   NOTE: Do NOT mix by inverting the tube to avoid blood accumulation in the cap and subsequent seepage during centrifugation.
4. Add 13 ml of Ficoll-paque (see **Materials** and **Equipment table**), to a new 50 ml capacity conical tube. Keep the tube upright in the rack.
5. Using a transfer pipette, take diluted blood, touch the transfer pipette tip to the inside wall of the tube near the top. With slow and steady pressure, transfer the blood along the inside wall of the tube forming a blood layer on top of the sucrose layer. Do this step several times until all blood has been transferred.
6. Cap the tube tightly and centrifuge the tube at room temperature for 30 min at 700 x g in a swing rotor with medium acceleration set to 5, and deceleration set to ZERO.
7. Slowly take out the tube and without disturbing the layers, take it back to the biosafety cabinet.
8. Carefully collect the buffy coat (thin cloudy white layer), where PBMCs are located, placed between the PBS/plasma and ficoll layers using a transfer pipette. Avoid collecting sucrose layer and do not disturb the red blood cell layer.
9. Transfer the buffy coat into a new 50 ml sterile conical tube. It may take several times of repeating **step 1.8** to collect all the PBMC.
10. Add PBS pH 7.4 to the tube with the PBMC and fill up to the 45 ml mark. Shake tube thoroughly. Do not vortex.
11. Wash 1: Centrifuge for 15 min at 480 x g with both maximum acceleration and deceleration set to 9. Observe formation of a pellet of PBMCs at the bottom of the conical tube.
12. Discard the PBS (supernatant) into a plastic beaker with 10 ml of bleach.
13. Loosen cell pellet by gently “racking” against an undulated surface (*i.e.,* empty rack). Do not vortex.
14. Add 25 ml of fresh PBS pH 7.4 to the tube. If more than one tube is being processed, pool the pellets together in this step.
15. Wash 2: Centrifuge the tube for 12 min at 480 x g with both maximum acceleration and deceleration set to 9.
16. Discard the PBS (supernatant) into the plastic beaker with a splash of bleach.

17. Gently "rack" against an undulated surface (*i.e.,* empty rack). Do not vortex.
18. Add 25 ml of fresh PBS to the tube.
    1. Take out 50 µl of the cells and transfer into a microcentrifuge tube for viability count.
    2. Add an equal amount (50 µl) of trypan blue (a viability stain) and pipette up and down to mix gently.
19. Take out 10 µl and transfer it to a hemocytometer to check the viability of cells per ml. Record the number of cells/ml.
20. Take out the desired amount of cells and centrifuge the tube for 12 min at 480 x g with both maximum acceleration and deceleration set to 9.
21. Discard the PBS (supernatant) into the beaker with bleach.
22. Gently "rack" against an undulated surface (*i.e.,* empty rack). Add 80 µl of PBS into the tube.

2. Making the PBMC Slide

1. Place 80 µl of the cells onto a microscope slide. Smear the drop with the help of another slide or place 2 drops of 40 µl each on both ends of the slide.
2. Leave slide in the biological safety cabinet to dry. Label the slide with a pencil on the frosted side.

3. Fixing the Samples on the Slides

1. Prepare the fixative solution by mixing 0.5 ml of 37% formaldehyde to 4.5 ml of 99% ethanol.
2. After the slides are dried, take out 2 ml of the freshly made fixative solution and pour it on the slide so that the entire surface of the slide is covered.
3. Leave the solution on the slide for 1 min. Rinse the slide for 1 min with tap water and leave it to air dry.

4. Making the Amylase Solution for Negative Control

1. Take 0.25 g of amylase powder and dissolve in 50 ml of distilled water. Pour the solution in a clean 100 ml beaker.
2. Immerse the slide in the beaker so that half of the slide receives the treatment and the other half remains untreated and then incubate for 15 min at room temperature (**Figure 1C**).
3. Make a note on which side of the slide is receiving the amylase treatment. Draw a line on the back of the slide indicating the border between the treatment and control.
4. Wash the slides with ddH_2O to remove the amylase solution and leave the slide to air dry.

5. Perform Periodic Acid Schiff (PAS) Staining and Imaging

NOTE: PAS reagents are toxic by inhalation and are corrosive, so the steps need to be done in a chemical fume hood, and the waste products must be properly disposed of according to institutional guidelines.

1. Place the slide on a flat surface and pour 1.50-2.00 ml of Periodic Acid Solution on the sample. Incubate for 5 min at room temperature.
2. Rinse the slides in several charges of distilled water.
3. Pour 1.50-2.00 ml of Schiff's reagent on the slide and incubate it at room temperature for 15 min.
4. Wash the slide with distilled water for 5 min and leave it to air dry.
5. Apply 10 µl mounting media on the slide and cover with two small coverslips, or apply 50 µl and use one large coverslip.
6. Apply clear nail polish on the edges of the coverslip, let dry overnight.

6. Obtain Images with the Binocular Light Microscope Using the 100X Objective

Representative Results

To validate the reagents and basic technique, PAS staining was performed according to manufacturer's instructions on mouse soleus muscle sections. The staining was done same day as the sacrifice and in the last step of the staining, the sections were fixed by xylene. The soleus muscle is known to contain ~35% glycogen-positive cells[9]. The stained muscle cells displayed two distinct PAS-positive features- punctate granules within the cell, and a continuous line demarking the cell membrane (**Figure 2A**). The presence of punctate granules is consistent with glycogen stores, and was used to define a positive muscle cell. Treatment of samples with amylase removed the punctate granules which is consistent with them being glycogen (**Figure 2B**).

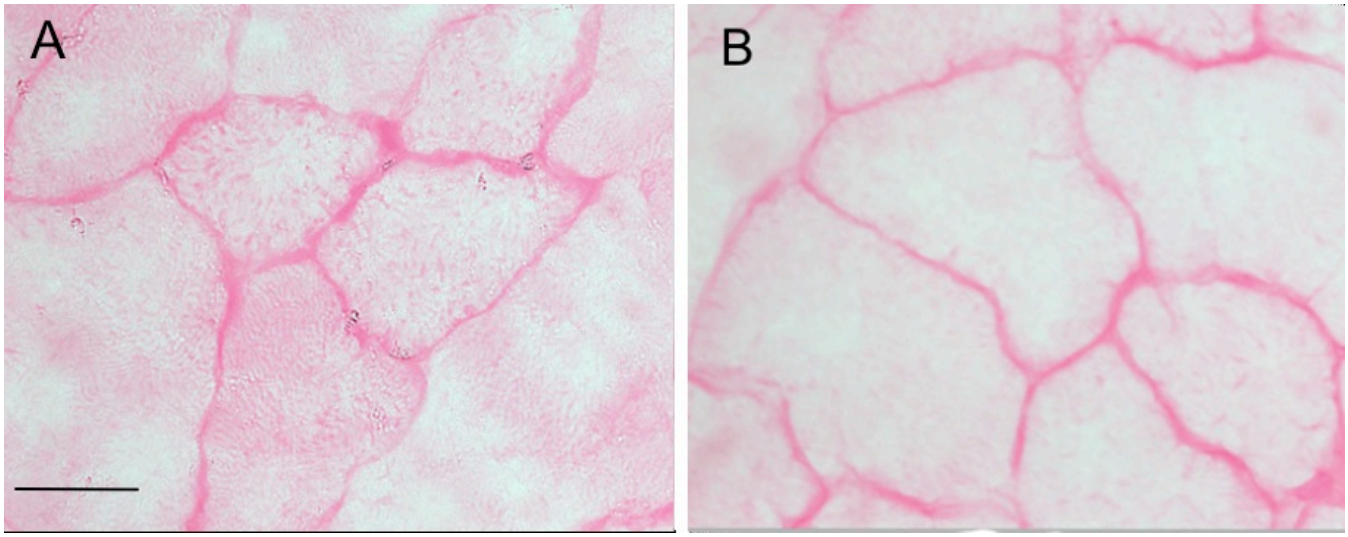

Figure 2: PAS-stained mouse muscle sections were analyzed with light microscopy. Mouse soleus muscle sections were stained with PAS. Some samples were pre-treated with amylase. **(A)** PAS-positive particles were visible inside of muscle cells. **(B)** Less PAS positive cells were observed when the muscle sections were pre-treated with amylase. The staining around the cell membrane remained after amylase treatment (scale bar = 100 µm). Please click here to view a larger version of this figure.

The membrane-staining signal remained even after amylase treatment. As expected, the percentage of PAS-positive cells in the non-amylase muscle section was 37%, while amylase treated muscle sections had significantly less, approximately 5% PAS-positive cells (**Figure 3**).

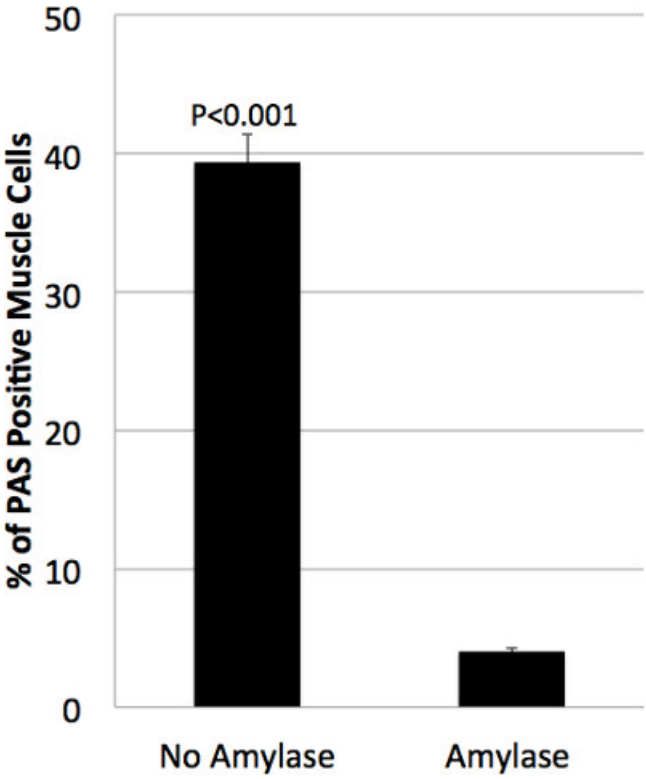

Figure 3: Quantification of PAS-positive muscle cells. The proportion of muscle cells that were PAS positive was counted from a representative slide without or with the amylase pre-treatment. As expected, 37% of muscle cells were positive for glycogen. Amylase treatment significantly reduced the PAS-signal to 4% (*p <0.001).

Next, PAS-staining was performed on PBMC from venous blood of healthy human subjects using a modified technique as described in the protocol section, and illustrated in **Figure 1**. The PBMC adhered well if the washing steps were carefully performed. The PAS-stained PBMCs displayed a variety of staining patterns. Small cells (5 µm) with granules were readily observed. A proportion of larger cells with a diffuse staining pattern were also observed (**Figure 4A**, and inset **A.1-6**). Treatment of samples with amylase removed the granule signal and diminished the diffuse PAS-positivity (**Figure 4B**).

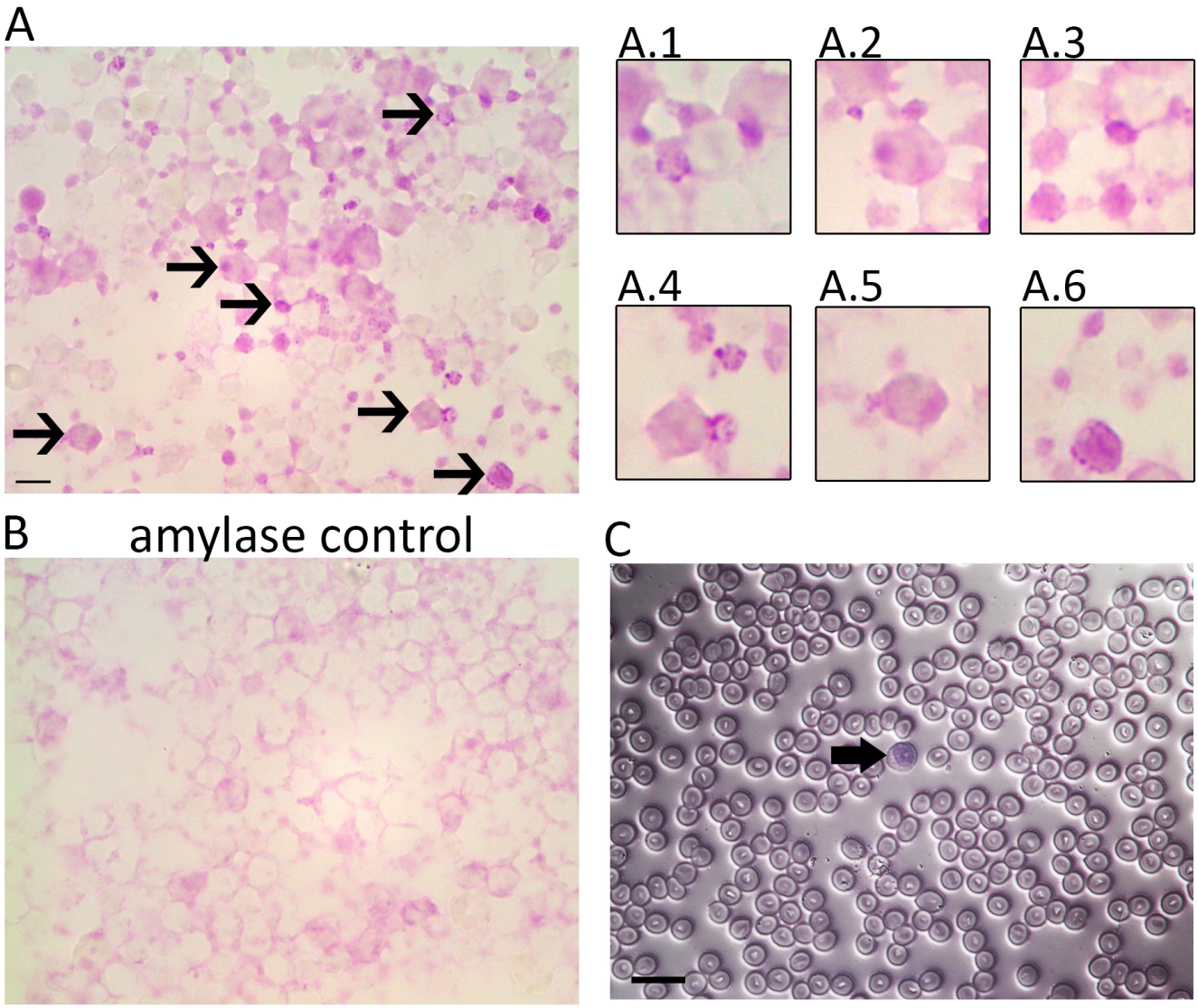

Figure 4: Periodic acid-Schiff (PAS) staining on human PBMCs. (A) PAS-staining on PBMCs was performed. Two types of cells were observed. (**A.1-6**) Smaller cells (at 5 µm) in non-amylase treated slides showed magenta particles consistent with glycogen. These cells could be resting cells. Larger cells (more than 5 µm) in non-amylase treated cells had diffuse PAS-positive staining. These cells could be activated lymphocytes. **(B)** PBMCs were treated with amylase for 15 min prior to staining, which diminished the PAS signal. Representative of seven different healthy human subjects. **(C)** The PAS and hematoxylin staining done on whole blood slide. The arrow shows a PBMC surrounded by many erythrocytes (scale bar = 10 µm). Please click here to view a larger version of this figure.

The proportion that was PAS positive was 98% for the smaller cells and 40% for the larger cells. Amylase treatment eliminated the PAS signal in the small cells ($p < 0.001$) and significantly diminished the PAS-signal in the larger cells to 7% (**Figure 5**).

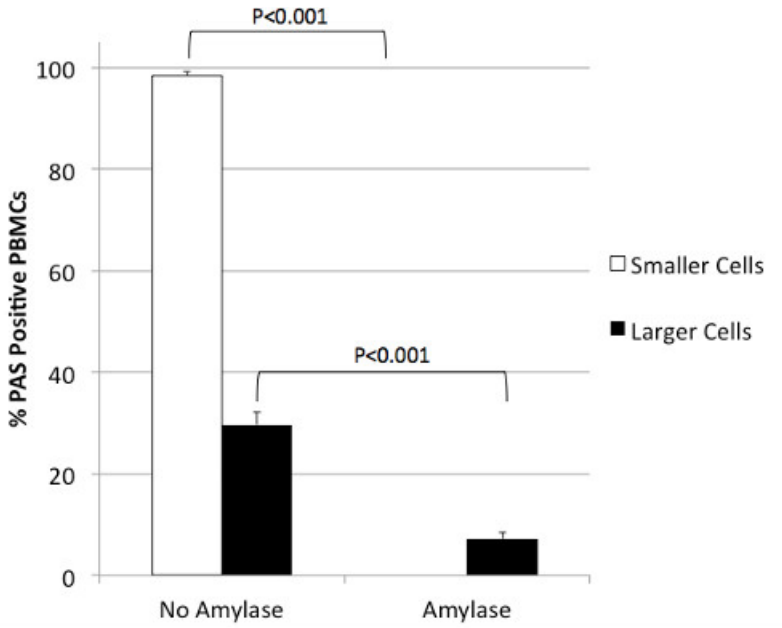

Figure 5: Quantification of PAS-positive PBMCs. The proportion of PBMCs that were PAS positive was counted from a representative slide without or with the amylase pre-treatment. 98% of the small-sized cells were positive for PAS. 40% of the larger cells were positive for PAS. Amylase treatment eliminated the PAS signal in the small cells ($p < 0.001$) and significantly diminished the PAS-signal in the larger cells to 7% ($p < 0.001$).

To confirm that PBMCs possess glycogen a second, independent method that also provides quantitation of the amount of glycogen was used[10,11], and has been previously published in JoVE on other cell types[12]. PBMCs were lysed in hypotonic buffer and glycogen was separated as described briefly in caption of **Figure 6**.

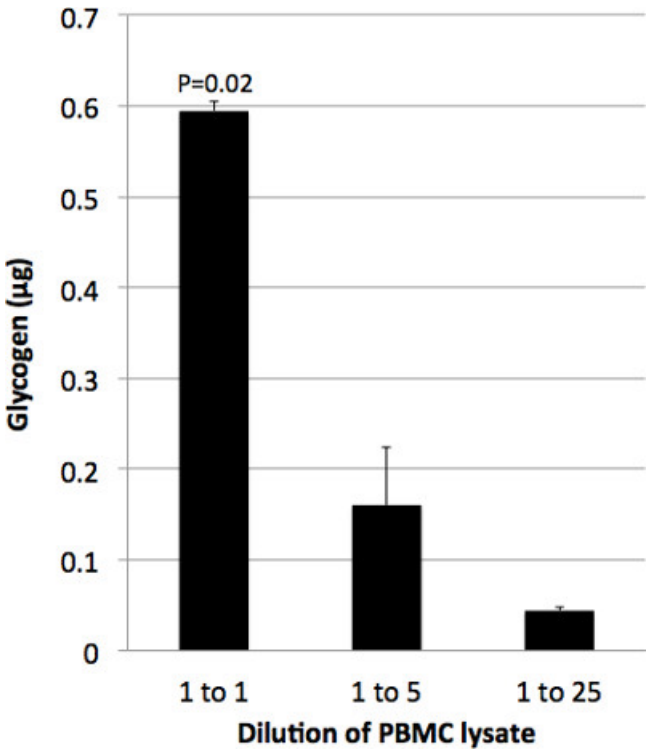

Figure 6: Measurement of glycogen using enzymatic digestion with hydrolysis enzymes. Glycogen was measured according to manufacturer's instructions. In brief, the protocol entails hypotonic lysis of 1 x 10^6 PBMCs followed by pelleting of insoluble material that contains glycogen. The pellet was washed and digested with hydrolysis enzymes yielding glucose which was measured by spectrophotometry and compared to a standard curve. The cell lysate was diluted at the indicated ratios with hydrolysis buffer. Data is representative of three experiments. Significant levels of glycogen were detected in the 1:1 dilution ($p = 0.02$), and this signal titrated out as the lysate was further diluted.

The insoluble glycogen was washed several times, and then digested using hydrolysis enzymes. The amount of glucose yielded from digestion was measured using spectrophotometry and compared to a standard curve. One million PBMCs had 1.19 µg of glycogen. The glycogen signal titrated down as the cell lysate was further diluted (**Figure 6**).

Discussion

The critical steps of this video article were during washing and amylase treatment of the cells. While washing the slides, the key step was using a plastic squeezable washing bottle and letting the water gently run through the sample on the slide and not aiming directly onto the samples. Even the slightest direct water pressure would cause the cells to come off the slide. Another key step was to use the same slide for ± amylase conditions. After the PBMCs were adhered to the slide, the slide was carefully placed into a beaker so only half of the smear was exposed to the amylase solution. This step provides a robust control because the blood cells are from the same slide, thus minimizing confounding variables that may occur due to slight timing variations. The cells stuck well with no treatment, so the additional cost and time involved with poly-L-lysine or polyethylene glycol coating for example would not be warranted.

Through troubleshooting the optimal activity of amylase was determined. For muscle sections, it was noted that the optimal activity of amylase was observed within 1 hr of incubation. At longer times the muscle sections would slowly peel off the slide, while at shorter times amylase did not adequately remove the PAS-signal. The timing for the amylase incubation for PBMC slides had to be reduced relative to the muscle-sample timing (which was 1 hr) down to only 15 min. Longer times caused PBMCs to come off the slide, while shorter times did not effectively impact the PAS-signal. One modification to the standard protocol of PAS staining was changing the method of washing the slides. Manufacturer's instructions indicated to wash the slides with running tap water, which caused the cells to come off the slides. The modification was to wash the slides with squeezable washing bottle to preserve the cells. As described in the critical steps section, it was very important not to directly apply water pressure on the cells.

There are some limitations in this technique. The muscle cell membrane was stained along with punctate granules in the cytoplasm of the muscle cell. The granules were eliminated by amylase treatment and therefore likely to be glycogen, while the membrane staining was insensitive to amylase. The identity of the PAS-positive particles on the cell membrane is not known. It could be the muscle basement (perimysium) membrane. This membrane surrounds the muscle fascicles and due to its high glycoprotein content it is known to be PAS positive. Another limitation of PAS staining is that glycogen granules must be at least 50 nm in diameter to be visible by conventional light microscopy. Thus, smaller glycogen granules could be present in a cell, but still register negative on a PAS test. The second method used overcomes this limitation by providing detection of glycogen in a lysate. In combination with a standard curve this can be used to determine the exact amount of glycogen. Although this technique is highly quantifiable and is not limited by the size of granules, it does itself have certain limitations. Glycogen granules are complex branched structures with many accessory proteins and chemical cross-links, making it unlikely that hydrolysis enzymes fully dissolve an entire glycogen molecule[13]. Thus, the enzymatic-detection (like the PAS technique) may under-represent the actual amount of glycogen in a sample. One way to handle this limitation is with electron microscopy which resolves even the smallest glycogen granules[14]. One would have to employ a combination of these techniques, PAS-staining, enzymatic digestion, and electron microscopy for the most comprehensive characterization of glycogen.

This article and video demonstrates the periodic acid Schiff (PAS) staining technique adapted for the use on PBMCs. The significance of this study is seen in the choice of using PBMCs over blood smear, which made it more feasible to enumerate lymphocytes. Initially, a classic blood-smear technique was tested, however the majority of cells on the slide were red blood cells and possibly neutrophils (**Figure 4C**). To concentrate the lymphocytes, PBMCs were purified from blood using standard density gradient technique. Using a technique similar to a blood-smear, the PBMCs readily adhered for the duration of the PAS procedure, which has many vigorous washing steps. The PAS technique has been used for decades to determine glycogen levels in muscle tissue biopsies, which are cut into thin sections and adhered to slides. PAS staining was chosen over other carbohydrate staining chemicals due to its high reliability and the presence of expected results in literature. A mouse (*Mus spretus*) muscle sections was used as a positive control and found, as expected, that 37% of cells were PAS-positive[9]. In terms of cost and time, preparing PBMC is more onerous than a blood smear, but there are several advantages. Firstly, the preparation is more enriched in lymphocytes, which are the cells of interest for projects that center on autoimmunity. If blood smears were used, lymphocytes would be the minority, making it challenging to find the cell of interest. One would have to prepare and analyze far more slides to get the same numbers you would get with a few PBMC slides. Researchers would be very interested in using our new optimized technique in autoimmunity projects related to Type 1 diabetes, multiple sclerosis, lupus, rheumatoid arthritis, *etc.* where T and B lymphocytes and NK cells play a role. Of course, for other applications where erythrocytes or neutrophils are of interest the blood smear would be recommended. The other advantage is that PBMCs can be used for studying human lymphocyte biology *in vitro*.

An ongoing research is investigating the source of glycogen in PBMCs. In the future, it is planned to measure glycogen content in the context of multiple sclerosis, a T lymphocyte-mediated autoimmune disease that affects an estimated 2.3 million people worldwide as of 2013[15,16]. What are the small and large cells in the PBMC samples? Cells at 5 µm are consistent with the lymphoid lineage in their resting state. T lymphocyte, B lymphocytes, natural killer cells, and other minor subsets are within this size range. The larger PBMCs are likely composed of activated lymphocytes, which grow bigger as they receive inflammatory signals from the immune system, and monocytes from the myeloid lineage. Advanced cell sorting techniques such as fluorescent activated cell sorting or magnetic-activated cell sorting are required to further refine the subset that expresses glycogen.

Hematoxylin counter staining was used when the staining was done on whole blood. This enabled us to distinguish monocytes from erythrocytes (**Figure 4C**). When the staining was done on PBMCs, since all cells were mononuclear, there was no need to counter stain with hematoxylin to identify cells. Also hematoxylin was interfering with the PAS signal in the cells. In summary, we have demonstrated a PAS-staining procedure adapted for PBMCs. This technique is useful for scientists and clinicians researching autoimmunity, infection and allergy.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This research was supported by a grant from the NSERC Discovery program grant number RGPIN 418522-2013. We thank R. Kilgour for helpful discussions, and Katelin Gresty and Dr. A. Berghdal for providing the mouse muscle sections.

References

1. Rich, P.R. The molecular machinery of keilin's respiratory chain. *Biochem.Soc.Trans.* **31** (Pt 6), 1095-1105, (2003).
2. Peter, J.B., Barnard, R.J., Edgerton, V.R., Gillespie, C.A., Stempel, K.E. Metabolic profiles of three fiber types of skeletal muscle in guinea pigs and rabbits. *Biochemistry.* **11** (14), 2627-2633, (1972).
3. Jones, R.V, Goffi, G.P., Hutt, M.S.R. Lymphocyte glycogen content in various disease. *J.Clin.Pathol.* **15** (1), 36-39, (1962).
4. Scott, R.B. Glycogen in human peripheral blood leukocytes. I. characteristics of the synthesis and turnover of glycogen in vitro. *J.Clin.Invest.* **47** (2), 344-352, doi:10.1172/JCI105730 (1968).
5. Fedele, D.. *et al.* P.A.S. positive index of lymphocytes and metabolic control in insulin-treated and type II diabetes mellitus. *Diabete Metab.* **9** (3), 188-192, (1983).
6. Brelińska-Peczalska, R., Mackiewicz, S. Cytochemical studies of peripheral blood granulocytes and lymphocytes in patients with systemic lupus erythematosus. *Pol.Med.Sci.Hist.Bull.* **15** (2), 231-234, (1976).
7. Yunis, A.A., Arimura, G.K. Enzymes of glycogen metabolism in white blood cells. I. glycogen phosphorylase in normal and leukemic human leukocytes. *Cancer, Res.* **24**, 489-492, (1964).
8. Hagemans, M.L., *et al.* PAS-positive lymphocyte vacuoles can be used as diagnostic screening test for pompe disease. *J.Inherit.Metab.Dis.* **33** (2), 133-139 doi: 10.1007/s10545-009-9027-4 (2010).
9. Totsuka, Y., *et al.* Physical performance and soleus muscle fiber composition in wild-derived and laboratory inbred mouse strains. *J.Appl.Physiol.* **95** (2), 720-727, doi: 10.1152/japplphysiol.00946.2002 (2003).
10. Murat, J.C., Serfaty A. Simple enzymatic determination of polysaccharide (glycogen) content of animal tissues. *Clin.Chem.* **20** (12), 1576-1577, (1974).
11. Arrizabalaga, O., Lacerda H.M., Zubiaga A.M., Zugaza J.L. Rac1 protein regulates glycogen phosphorylase activation and controls interleukin (IL)-2-dependent T cell proliferation. *J.Biol.Chem.* **287** (15), 11878-11890, doi: 10.1074/jbc.M111.297804 (2012).
12. Pelletier, J., Bellot G., Pouysségur J., Mazure, N.M. Biochemical titration of glycogen in vitro. *J.Vis.Exp.* **(81)**, doi: 10.3791/50465 (2013).
13. Roach, P.J., Depaoli-Roach A.A., Hurley T.D., Tagliabracci V.S. Glycogen and its metabolism: Some new developments and old themes. *Biochem.J.* **441** (3), 763-787, doi: 10.1042/BJ20111416 (2012).
14. Salmoral, E.M., Tolmasky D.S., Krisman C.R. Evidence for the presence of glycogen in rat thymus. *Cell Mol.Biol.* **36** (2), 163-174 (1990).
15. Darlington, P. J., *et al.* Diminished Th17 (not Th1) responses underlie multiple sclerosis disease abrogation after hematopoietic stem cell transplantation. *Ann.Neurol.* **73** (3), 341-354, doi: 10.1002/ana.23784 (2013).
16. Pelletier, J., Bellot, G., Pouysségur, J., Mazure, N.M. *Multiple Sclerosis International Federation Atlas of MS.* http://www.atlasofms.org/ (2013).

