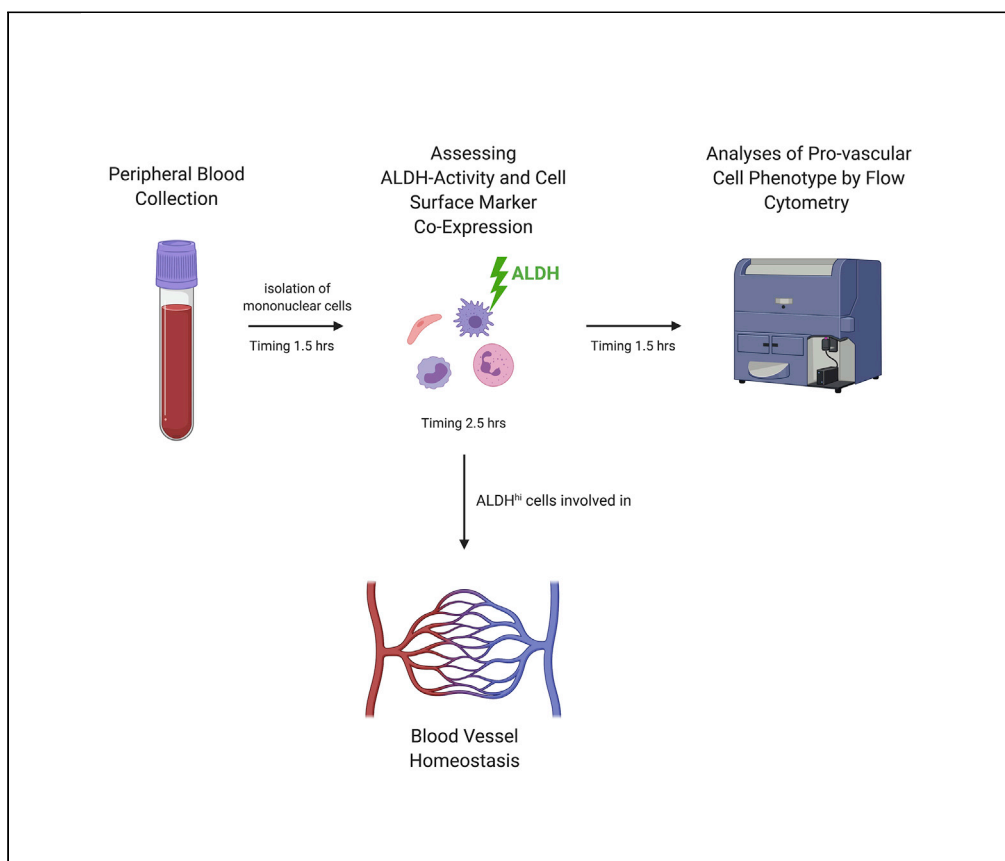# STAR Protocols

Protocol

Isolation and characterization of circulating pro-vascular progenitor cell subsets from human whole blood samples

The examination of circulating pro-vascular progenitor cell frequency and function is integral in understanding aberrant blood vessel homeostasis in individuals with cardiometabolic disease. Here, we outline the characterization of progenitor cell subsets from peripheral blood using high aldehyde dehydrogenase (ALDH) activity, an intracellular detoxification enzyme previously associated with pro-vascular progenitor cell status. Using this protocol, cells can be examined by flow cytometry for ALDH activity and lineage restricted cell surface markers simultaneously.

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HIGHLIGHTS
Aldehyde dehydrogenase is superior in the isolation of progenitor cells

Flow cytometry is an effective method to characterize pro-vascular cells

Aggressive gating strategies allows for in-depth progenitor cell characterization

The use of fresh blood samples will yield most accurate cell prevalence

Protocol

Isolation and characterization of circulating pro-vascular progenitor cell subsets from human whole blood samples

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SUMMARY

The examination of circulating pro-vascular progenitor cell frequency and function is integral in understanding aberrant blood vessel homeostasis in individuals with cardiometabolic disease. Here, we outline the characterization of progenitor cell subsets from peripheral blood using high aldehyde dehydrogenase (ALDH) activity, an intracellular detoxification enzyme previously associated with pro-vascular progenitor cell status. Using this protocol, cells can be examined by flow cytometry for ALDH activity and lineage restricted cell surface markers simultaneously.

For complete details on the use and execution of this protocol, please refer to Terenzi et al. (2019) and Hess et al. (2019, 2020).

BEFORE YOU BEGIN

Aldefluor for the assessment of ALDH activity

Aldefluor (fluorescent cell substrate of ALDH) is a means through which ALDH activity can be assessed using a single cell suspension from umbilical cord blood, bone marrow or from peripheral blood (Hess et al., 2004; Storms et al., 1999). Aldefluor consists of an active ALDH-substrate, an amino acetaldehyde molecule covalently bound to a BODIPY (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-5-proprionic acid) fluorochrome that is metabolized by ALDH to an aminoacetate anion. The anion is retained inside the cell due to its resultant negative charge. Therefore, the amount of fluorescent product that accrues in viable cells correlates directly to ALDH activity. Importantly, progenitor cells of several lineages consistently demonstrate high levels of ALDH activity (Figure 1. High aldehyde dehydrogenase (ALDH) Expression simulates progenitor cell differentiation). As cells differentiate toward maturity, ALDH activity is decreased. In order to quantify the level of ALDH activity within each viable cells, we compare the amount of the fluorescence product generated to cells treated with N,N-diethylaminobenzaldehyde (DEAB), a reversible inhibitor of ALDH activity. The
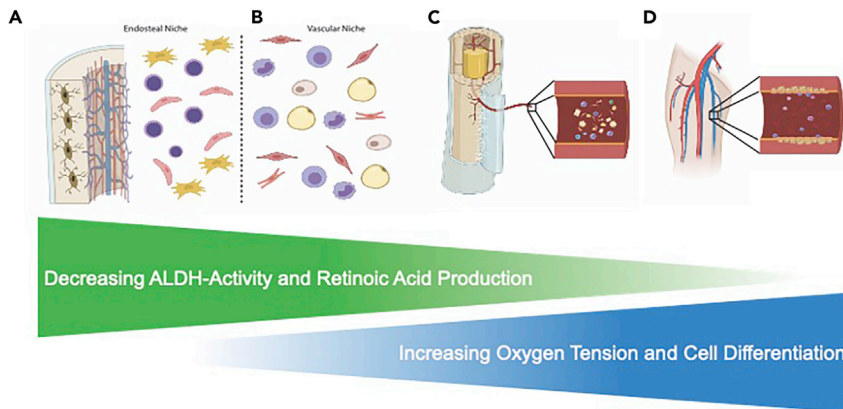
Figure 1. High aldehyde dehydrogenase (ALDH) expression simulates progenitor cell differentiation

final essential component of the Aldefluor kit is the ALDH buffer which impedes the transport of metabolized Aldefluor reagent out of target cells during cell sorting and analysis procedures.

Purpose of ALDH activity

Traditionally, the characterization of hematopoietic progenitor cells has been achieved using cell surface markers such as CD34 and CD133 (Fadini et al., 2006, 2005). Although surface markers can be used to loosely categorize progenitor cells of hematopoietic and endothelial lineages, these markers do not confer true progenitor cell status, and can fluctuate depending on cell cycle status and inflammatory conditions. Furthermore, there continues to be discrepancies in the literature concerning the most effective surface markers in predicting progenitor cell phenotype (Peichev et al., 2000; Isner and Asahara, 1999; Hur et al., 2004). In contrast to cell surface markers where the marker does not confer a specific progenitor cell function, ALDH is an intracellular enzyme that protects immature cells from oxidative stress and alkylating agents such as cyclophosphamide (Hess et al., 2004; Storms et al., 1999). Thus, long lived progenitor cells with high ALDH activity are resistant to oxidative damage. ALDH also represents also the rate limiting enzyme in the production of retinoic acid, a potent morphogen that drives progenitor cell expansion and differentiation in multiple lineages (Figure 1). As primitive progenitor cells mature and egress from the bone marrow, the expression of ALDH, is expected to diminish such that few circulating cells will possess high ALDH activity (Figure 1). However, using highly sensitive FACS technology, we can characterize and isolate cells with high ALDH activity that correlates consistently with progenitor status and function (Hess et al., 2006; Putman et al., 2012).

Our lab has demonstrated the utility of detecting cells with elevated ALDH activity in hematopoietic and endothelial progenitor cells with pro-vascular secretory function and functional vessel forming capacity *in vivo* and *in vitro* (Putman et al., 2017; Capoccia et al., 2009; Hess et al., 2006; Putman et al., 2012). Cells from BM or umbilical cord blood (UCB) with elevated ALDH activity demonstrated increased rates of multipotent hematopoietic colony formation when compared to cells with low ALDH activity. Furthermore, endothelial cells with high ALDH activity demonstrated enhanced tubule forming capacity in Matrigel. In animal models of surgically induced critical limb ischemia, injection of cells with elevated ALDH activity increased limb perfusion and limb use compared to cells with low ALDH activity via the stimulation of collateral small vessel formation. Proteomic analyses have revealed that cells with elevated ALDH activity possess a pro-vascular secretory profile and accelerate recovery of perfusion via neo-vessel formation induced by paracrine effects (Capoccia et al., 2009; Cooper et al., 2018; Putman et al., 2012, 2017). Finally, we have examined ALDH activity in the characterization of pro-vascular progenitor cells in humans with T2D, undergoing bariatric surgery and receiving sodium glucose co-transporter 2 (SGLT2) inhibitors (Terenzi et al., 2019; Hess et al., 2019, 2020). Notably, ALDH activity assessment in cells from peripheral blood was established as an important biomarker to discern pro-vascular progenitor cells in the peripheral vasculature and

ALDH assessment can be used as an important tool to detect progenitor cells that support blood vessel homeostasis.

General guidelines and preparations

1. 20 – 30 mL of whole blood should be drawn by venipuncture and collected in 10 mL BD lavender tubes. These tubes are coated in EDTA and limit coagulation of peripheral blood. Following collection, tubes should be inverted several times. Blood samples should be processed within 24 h of collection.
2. Warm Ficoll Paque to room temperature (20°C) (10–15 min) before use.
3. Place SepMate collection tubes (15 mL) in the biological safety cabinet (BSC) based on how much blood you intend to process (1 SepMate/5 mL of blood collected).
4. Ensure pipette aid is sterilized and placed in BSC along with 10 mL serological pipettes to layer up to 10 mL of whole blood mixture onto Ficoll Paque.
5. Ensure full biocontainment and sterile procedures are followed.

KEY RESOURCES TABLE

| REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD133 (APC)	Miltenyi	130-090-826
Anti-human CD34 (PE)	BioLegend	343606
Anti-human CD45 (PerCP-Cy5.5)	BioLegend	368604
Anti-human CD163 (PE-Cy7)	BioLegend	366618
Anti-human CD86 PerCP-Cy5.5	BioLegend	374216
Anti-human CD31 (PE-CY7)	BioLegend	303118
Anti-human CD33 (PE-Cy7)	BioLegend	366618
Anti-human CD144 (APC)	BioLegend	348508
Anti-human CD146 (BV421)	BioLegend	361015
Anti-human CXCR4 (BV421)	BioLegend	306518
Anti-human CD14 (PE)	BioLegend	367104
Anti-human CD14 (PE-Cy7)	BioLegend	557742
Anti-human CD206 (APC)	BioLegend	321110
Anti-human CD15 (PE)	BioLegend	301906
Anti-human CD16b (PE-Cy7)	BioLegend	550868
Anti-human CD66b (APC)	BioLegend	305118
Commercial assays		
Ammonium chloride lysis buffer	STEMCELL Technologies	07850
SepMate PBMC isolation tubes	STEMCELL Technologies	85415
Aldefluor kit	STEMCELL Technologies	01700
Aldefluor assay buffer	STEMCELL Technologies	01702
Ficoll Paque Plus	GE Healthcare	36-101-6383
Other		
Falcon (50 mL) conical centrifugation tubes	BD Biosciences	352070
Falcon (5 mL) polystyrene tubes	BD Biosciences	352054
Falcon serological pipettes (10 mL)	BD Biosciences	357551
Portable Pipet Aid	Drummond Scientific	703-350-02
Pasteur pipette	Fisherbrand	13-678-20A
Transfer pipette	STEMCELL Technologies	38086
Trypan blue dye	Thermo Fisher Scientific	15250061
Hemocytometer	Hausser Scientific	1490
Eppendorf tube (0.5 mL)	Eppendorf	0030121023
Fetal bovine serum	Sigma-Aldrich	F2442
Phosphate buffered saline	Gibco	10010023
UltraComp eBeads (compensation beads)	Thermo Fisher Scientific	01-3333-42
FC precision counting beads	BioLegend	424902
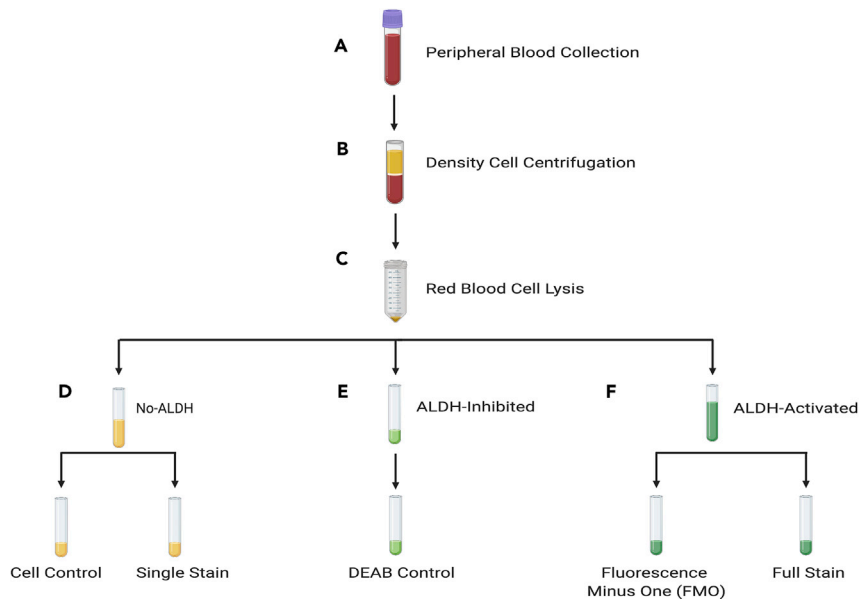
Figure 2. Mononuclear cell isolation and staining for ALDH activity and lineage restricted cell surface markers

Alternatives: All of the cell culture equipment can be substituted with similar products from various companies.

STEP-BY-STEP METHOD DETAILS
Preparation of peripheral blood mononuclear cells

⏱ Timing: 1.5 h

Figure 2 "Mononuclear cell isolation and staining for ALDH activity and lineage restricted cell surface markers" provides a schematic through which this experiment is summarized.

1. Place up to 25 mL of collected whole blood in a 50 mL Falcon tube.
   a. Dilute blood sample 1:1 with phosphate buffered saline (PBS) and mix thoroughly.
   b. We recommend using a pipetting controller on a slow setting with 10 mL serological pipettes.
2. 15 mL collection tubes
   a. Fill 15 mL SepMate (STEMCELL Technologies, Vancouver, BC) tube with 4 mL of room temperature (20°C) Ficoll Paque. Place serological pipette tip in the SepMate insert groove to guide the Ficoll (Figure 3A. Visual Representation of Sample Preparation),
      i. Note that this can be done prior to beginning the experiment. You may find it preferable to prepare SepMate tubes in advance and place them in the fridge. This will reduce time needed for the Ficoll to reach room temperature (20°C).
3. Layer up to 10 mL of whole blood + PBS solution into the SepMate collection tubes (Figure 3B) for density centrifugation.
   a. We recommend the slowest setting on the pipette controller to gently layer the whole blood + PBS mixture onto the Ficoll.
   b. Layer the blood while the SepMate tube is at a slight angle. This will limit blood and Ficoll mixing and will improve separation.
4. Centrifuge the sample at 1,500 rpm (approximately 425 × *g*) for 10 min at room temperature (20°C) with the break ON.
   a. If unable to use SepMate system, follow the same procedure, but the sample will need to be centrifuged for 20 min with the break OFF. This will add significant time to cell processing.
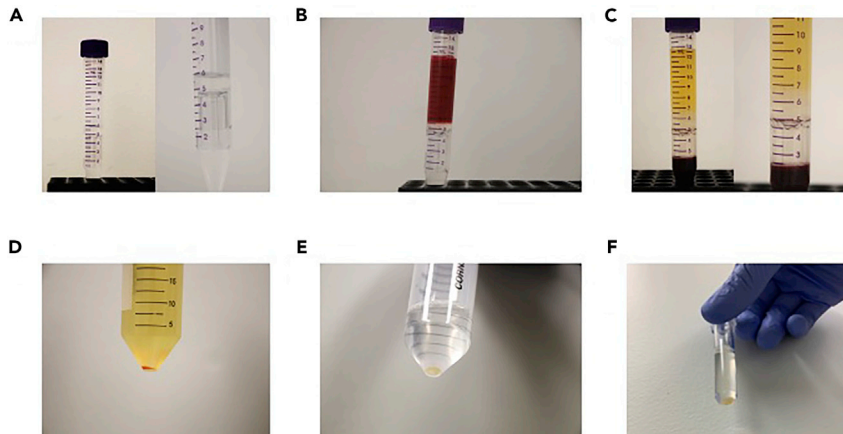
Figure 3. Visual representation of sample preparation

    b. Ensure that the centrifuge is balanced properly to eliminate damage to the cells or centrifuge.

5. Remove sample from centrifuge and collect the fluid above the SepMate insert (Figure 3B) and place into a sterile 50 mL Falcon tube.

    a. SepMate allows for direct pour-over of contents, the SepMate insert will keep pelleted debris and red blood cells from contaminating the mononuclear cell layer.

    b. If not using SepMate, removal of buffy coat should be done using a transfer pipette, with slow manual swirling. User should be very careful to not disturb the red blood cell pellet (See Figure 3C).

    c. Discard used SepMate tubes to maintain a clean workspace. (see Troubleshooting 1)

6. Dilute the mononuclear cell sample 1:1 with PBS + 5% FBS (fetal bovine serum).

    a. The growth factors found in FBS maintain cell homeostasis and limit cellular damage due to changes in solution tonicity.

    b. We recommend preparing this solution using a full bottle (500 mL) of sterile PBS and then adding 5% (25 mL) of filtered FBS solution. This solution should be properly labeled and placed in a 2°C–8°C fridge for future use.

7. Centrifuge the sample at 1,500 rpm (approximately 425 × g) for 7 min with the break ON

8. Remove the supernatant and resuspend cell pellet in 1 mL PBS + 5% FBS followed by addition 9 mL of ammonium chloride (NH_4Cl) red cell lysis buffer. (see Troubleshooting 2)

9. Let the sample rest at room temperature (20°C) for 8 min to ensure red cell lysis.

10. Centrifuge the sample at 1,500 rpm (approximately 425 × g) for 7 min with the break ON.

    a. If any pink or red coloring remains in the pellet following centrifugation (Figure 3D) of the sample, repeat steps 8–10 to maximize erythrocyte depletion (Figure 3E). This repeat can be done up to 2 times, for a total of 3 rounds of red blood cell lysis.

    b. Remove the supernatant and resuspend the pellet by washing with 3 mL of Aldefluor buffer assay solution (STEMCELL Technologies, Vancouver, BC)

    c. Single cell suspension should appear cloudy with disruption of the pellet.

    d. If the pellet does not dissolve completely and appears as a suspension, you may choose to add more Aldefluor Assay Buffer to ensure proper emulsion. If proper emulsion does not occur, samples may become clogged in the flow cytometer or the cell sorter which may result in loss of sample or other technical difficulties.

11. Pipette approximately 20 μL of mixed sample into a 0.5 mL Eppendorf tube.

12. Into the same Eppendorf, add 20 μL of Trypan blue and mix thoroughly to assess cell numbers and viability.

13. Add 10 μL of the cell solution +Trypan mixture to each hemocytometer chamber and count the viable (clear) and non-viable cells. Cell viability should be >90%. The usual PBMC yield is approximately 1 × 10^6 cells per mL of peripheral blood drawn. If the cell viability is <90% ensure

proper mixture of the cell solution prior to addition of trypan blue. If this does not rectify the cell count, it is possible that that sample is no longer viable and phenotypic analyses may not be accurate.
   a. The use of automated cell counting methods are also applicable.
   b. Adjust the sample to a concentration of 1 × 10^6 cells/mL with Aldefluor Assay Buffer.

Note: Use of Aldefluor buffer is essential for subsequent steps as it contains a proprietary inhibitor of ATP-binding cassette pumps allowing for accumulation of Aldefluor substrate within viable cells.

Purification of pro-vascular progenitor cells by ALDH activity

⏱ Timing: approx. 1.5 h

14. Label 5 mL polystyrene Falcon tubes according to various surface markers and controls you will be examining.
   a. Label one tube *"no-ALDH,"* this will be divided into tubes allocated for the unstained control and each fluorescence isotype.
   b. Label one tube *"ALDH-inhibited,"* this will be used to evaluate ALDH-inhibited controls using DEAB.
   c. Label one tube *"ALDH-stained,"* this will contain cells with activated aldehyde dehydrogenase and will be later divided into tubes for examining FMOs and antibody-stained aliquots.
15. Allow the Aldefluor reagent and kit components (STEMCELL Technologies, Vancouver, BC) to come to room temperature (20°C)
16. Remove an aliquot (we suggest 20 μL of previously activated Aldefluor substrate for each aliquot) from the freezer (−20°C) and allow to come to room temperature (20°C).

Note: to view activation of Aldefluor protocol and more details regarding product use, visit https://cdn.stemcell.com/media/files/pis/29888-PIS_1_1_2.pdf

17. We recommend separating the cell solution into several 5 mL polystyrene tubes to reduce formation of sample clogs, however, the use of 5 mL polypropylene tubes are acceptable.
   a. Place 1 mL of the cell solution, which equates to approximately 1 × 10^6 cells/mL into a tube labeled *"no-ALDH."* Cap the tube and set it aside.
   b. Place 2 mL of the cell solution into an additional tube labeled *"ALDH-stained"* tube. This sample will act as the Aldefluor-stained positive control.

18. Add 5 μL of Aldefluor DEAB Reagent to the *"ALDH-inhibited"* (DEAB) tube.

⚠ CRITICAL: Recap DEAB vial immediately. If not, the DEAB will begin to evaporate and reduce inhibition potency.
   a. DEAB is a reversible pharmacological inhibitor of cytosolic ALDH activity. (see Troubleshooting 4)

19. Add 5 μL of the activated Aldefluor Reagent per mL of sample to the *"ALDH-stained"* tube.
   a. Mix and immediately transfer 500 μL of the cell mixture to the *"ALDH-inhibited"* (DEAB) tube.
   b. Mix gently with P1000 pipette.
20. Place *"ALDH-inhibited"* and *"ALDH-stained"* samples into a 37°C with 5% CO_2 incubator for 30–60 min.
21. Place *"no-ALDH"* tube at 2°C–8°C until the *"ALDH-stained"* and *"ALDH-inhibited"* samples are removed from the incubator.

Staining of pro-vascular progenitor cells

© Timing: approx. 1 h

22. Label a 5 mL polystyrene Falcon tubes according to single fluorochromes you will be examining.
    a. From your *"no-ALDH"* unstained control, add an equal sample volume into each fluorochrome labeled tube. For example, you will have one PE labeled tube, one PE-Cy7 labeled tube etc.
    b. If following the exact methodology using 6 fluorochromes, you will have 7 tubes in total (including the *"no-ALDH"* unstained control). This will equal approximately 125 μL into each tube.

    Note: There is no limitation to the number of cells needed to run flow cytometry, less sample, however, may result in fewer events of the desired cell population. We suggest a maximum of 6 fluorochromes to ensure ample cellular events.
    c. Keep any remaining sample in the *"no-ALDH"* unstained control.

23. Label a 5 mL polystyrene Falcon tubes for each fluorescence minus one (FMOs) control you will be examining.
    a. For example, if you are using 6 fluorophores, you will have 6 FMO tubes.
24. Label a 5 mL polystyrene Falcon tube for each fully stained panel you will be examining.
    a. For example, if you have 4 full panels, you will require 4 tubes.
    b. We suggest labeling each tube with the cell of interest. For example, "FULL-hematopoietic," "FULL-monocyte," "FULL-endothelial" etc.
25. After 30–60 min in the incubator, remove the *"ALDH-inhibited"* and *"ALDH-stained"* tubes from the incubator and mix the contents of each tube with a P1000 pipette (Figure 3F).
    a. From the *"ALDH-stained"* sample tube, allot an equal sample volume into each FMO and full stain tube.
    b. If following our exact methodology of 6 fluorochromes, you will have 10 tubes in total, including the "ALDH-stained" control (1 ALDH-stained control, 5 FMO, 4 full stained tubes). This will equal approximately 180 μL into each tube.
    c. Following aliquot, keep any remaining sample in the "ALDH-*stained*" *control.*
26. Centrifuge all the FMO tubes, full panel tubes, "ALDH-inhibited" and "ALDH-stained" control tubes at 1,500 rpm (approximately 425 × *g*) for 5 min with the break ON.
    a. The unstained *"non-ALDH"* tube and fluorochrome isotype tubes do not have to be centrifuged at this point.
27. Following the centrifugation, remove the supernatant of each tube described in step 26, and re-suspend the pellet in approx. 200 μL of Aldefluor Assay Buffer per sample tube using P200 pipette.

28. Add 4 μL each antibody to your test tube based on your specified panels (Figure 2) using a P10 or P20 micropipette.
    a. Note: do not dilute the antibodies.
    b. Following addition of all antibodies, mix gently with a P200 pipette to ensure proper mixture of the sample.

    ⚠ CRITICAL: change pipette tips when introducing each unique antibody to avoid contamination of your sample and antibody storage.
    c. For each fluorochrome isotype, place only the fluorochrome of interest. For example, only the PE-conjugated antibody in the PE isotype tube.
    d. For each FMO, place every fluorochrome except the one of interest. For example, everything but PE in the FMO-PE tube.
    e. For each full panel tube, place all antibodies specific to each cell panel.

Table 1. Flow cytometry tubes

Cell count - voltages	Tube contents
Unstained control	Only PBMNCs
Aldehyde Activity	ALDH-inhibited (DEAB)
	ALDH-activated
Compensation Controls	FITC Compensation
	PE Compensation
	PerCP-Cy5.5 Compensation
	PE-Cy7 Compensation
	APC Compensation
	BV421 Compensation
Single Stain Controls	PE Single Stain
	PerCP-Cy5.5 Single Stain
	PE-Cy7 Single Stain
	APC Single Stain
	BV421 Single Stain
Fluorescence Minus One	PE-FMO (Includes PerCP-Cy5.5, PE-Cy7, FITC, APC, BV421)
	PerCP-Cy5.5-FMO (Includes PE, PE-Cy7, FITC, APC, BV421)
	PE-Cy7-FMO (Includes PE, PerCP-Cy5.5, FITC, APC, BV421)
	APC-FMO (Includes PE, PerCP-Cy5.5, PE-Cy7, FITC, BV421)
	BV421-FMO (Includes PE, PerCP-Cy5.5, PE-Cy7, FITC, APC)
Full Panels	Full-Hematopoietic (ALDH, CD34, CD45, CD33, CD133, CXCR4)
	Full-Endothelial (ALDH, CD34, CD45, CD31, CD144, CD146)
	Full-Monocyte (ALDH, CD14, CD86, CD163, CD206)
	Full-Granulocyte (ALDH, CD15, CD14, CD16b, CD66b)

29. Place all samples in the fridge (2°–8°C) for at least 30 min. This will allow for appropriate antibody binding onto MNCs.

   Note: Antibodies can remain in the fridge for longer than 30 min, but we recommend a maximum of 2 h to ensure the analyses of fresh samples.

30. Following incubation, place all samples including *"non-ALDH"* unstained control in the centrifuge at 1,500 rpm (approximately 425 × *g)* for 5 min with the break ON.
31. Remove the supernatant and resuspend cells in approximately 300 μL of Aldefluor Assay Buffer using a P1000 pipette.

   ⚠ CRITICAL: Ensure that the pellet is properly dissolved in the solution. If the solution is too concentrated, it will appear cloudy. To remedy this, add more Aldefluor Assay Buffer until the sample is clear. If the sample is not properly diluted, clogging of the flow cytometer or cell sorter may occur, reducing the number of accurately processed events.

32. Cap the 5 mL polystyrene Falcon tubes and place in the fridge (2°C–8°C) until ready to be analyzed on the flow cytometer.

   Note: If unable to analyze on the cytometer or cell sorter immediately, we suggest leaving the prepared cells in the fridge for a maximum of 3 h. Also note that cells must be analyzed within 24 h of peripheral blood collection to ensure appropriate viability and expression of surface markers. (See Troubleshooting 3)

Preparing for flow cytometry
A typical flow cytometry panel of this nature contains (Table 1):

(1) an unstained control to optimize the laser voltages for the cell population of interest;
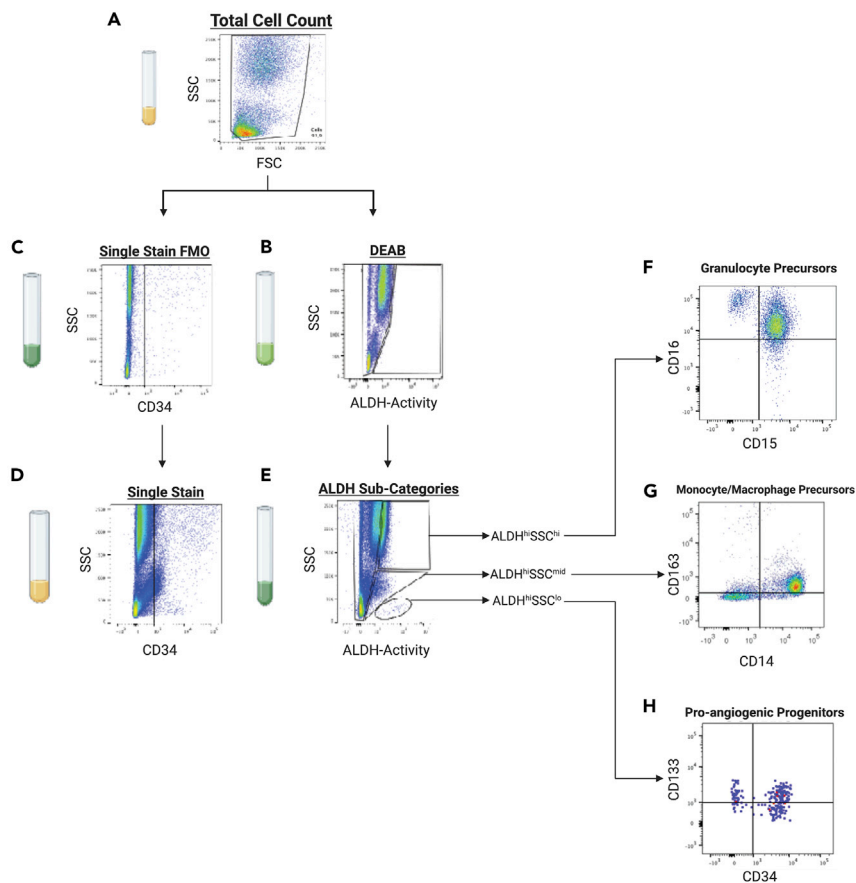
Figure 4. Sample workflow of precursor cell analyses by flow cytometry

(2) an isotype control for each fluorescence-labeled anti-human antibody (which will account for non-specific binding);

(3) a single-stained compensation control tube for each fluorescence-labeled anti-human antibody employed;

(4) tubes containing fluorescent anti-human antibodies that are used for final analyses.

We divide our antibody panels using cell surface markers expressed on 4 specific cell types including primitive progenitor, endothelial, monocyte precursors, and granulocytes. Each antibody is selected based on published literature and our lab's experience with these surface markers. Furthermore, our panels typically consist of six different fluorochromes including FITC, PE, PerCP-Cy5.5, PE-Cy7, APC, and BV421.

Running flow cytometry

⏱ Timing: approx. 1.5 h

Note: We typically do not filter our cells prior to flow cytometry as the addition of excess Aldefluor buffer is sufficient to remove clumps of cells that did not properly dissolve. If this technique does not remedy the formation of clumps, we suggest using a 35 μm mesh cap to filter the cells (Falcon 352235).

Below is the typical order through which our tubes are assessed. Please refer to Figure 4 "Sample Workflow of Precursor Cell Analyses by Flow Cytometry" for a schematic outlining a sample flow cytometry analysis.

33. Unstained control – will be used to establish forward and side scatter which examine cell size and granularity respectively. This allows for the effective characterization of cell morphology during analysis. Furthermore, from this panel we will gate on only cellular events, removing any excess debris or red blood cell contamination (these events have very low forward and side scatter values). We suggest recording 50,000 events to allow for precise gating of cellular events.

34. Compensation tubes – each tube will be specific for the fluorochromes that will be utilized. These tubes are used to optimize the compensation and to correct for any spectral overlap between fluorophores. We suggest recording 20,000 events per compensation tube to allow for accurate compensation.

35. DEAB control – will be used to create a positive and negative region for ALDH activity. This is especially important as we can confirm the immature phenotype of primitive progenitor cells. The gate should be set at < 0.5% and we suggest recording 50,000 events to allow for precise gating of DEAB inhibition.

36. ALDH activity – will be used to assess ALDH activity in PBMNCs. There should be clear separation of granulocyte precursor and mature cells as depicted in Figure 4. Furthermore, what we term ALDH^{hi}SSC^{low} cells should have the greatest ALDH activity as they are the most primitive cell type, as such, they will have a greater forward scatter value than all other cell types. We suggest recording 50,000 events to allow for precise gating of ALDH activity and subsequent cell populations. (see Troubleshooting 5)

37. Fluorescence minus one (FMO) tubes – will be used to establish gates for each fluorochrome in your panel. Each tube will contain all fluorochromes of interest but one. For example, the FMO for PE in our panel would contain FITC, PerCP-Cy5.5, PE-Cy7, APC, and BV421. We suggest recording 50,000 events for each FMO tube to allow for precise gating of each fluorophore.

38. Full panel analyses – is comprised of all surface marker antibodies in the specific panel you are testing. Since most analyses will come from these tubes, we suggest recording upwards of 1 million events to allow for appropriate examination of rare cellular events.

EXPECTED OUTCOMES

Through the combined use of the gating strategies specified, elevated ALDH activity, and lineage specific surface markers, we have established a high throughput assay that affectively characterizes granulocyte precursor, monocyte precursor, and pro-angiogenic progenitor cell prevalence in patient versus control populations.

Variability when analyzing human PB samples is to be expected. For example, individuals with increased risk of glucotoxicity, lipotoxicity and risk of ischemic cardiovascular complications are expected to have a reduced prevalence of pro-vascular progenitor cells with elevated ALDH activity (Terenzi et al., 2019; Hess et al., 2019). Moreover, in disease states, such as T2D, that confer elevated and/or chronic inflammation, we expect a decreased prevalence of monocyte precursor cells with elevated ALDH activity and an increased prevalence of pro-inflammatory granulocyte precursor cells with elevated ALDH activity compared to healthy controls.

Furthermore, we have demonstrated that true hematopoietic progenitor cells (ALDH^{hi}SSC^{low} CD34+/CD133+) are extremely rare (<0.05% of cellular events) in the peripheral blood. However, when using umbilical cord blood, you can expect the prevalence of progenitor cells with elevated ALDH activity to be considerably elevated (approximately 1% of cellular events).

Please refer to (Terenzi et al., 2019; Hess et al., 2019, 2020) for data related to human peripheral blood

Please refer to (Putman et al., 2017; Capoccia et al., 2009; Hess et al., 2006) for data related to umbilical cord blood data

QUANTIFICATION AND STATISTICAL ANALYSIS

1. We first gate on all cellular events, this is done to remove any remaining cellular debris that was detected (Figure 4A). Axes should be labeled as FSC and SSC on the X and Y axes respectively.
   a. Apply this gate to all plots to maintain a consistent gate shape for all tubes of interest.
2. Use *"ALDH-inhibited"* DEAB plots to establish a positive and negative gate for ALDH activity (Figure 4B)
   a. Label cells to the right of the DEAB gate as "ALDH^{hi}" cells and cells to the left of the DEAB gate as "ALDH^{lo}" cells.
   b. This allows the user to clearly decipher immature precursor "ALDH^{hi}" cells from mature and differentiated "ALDH^{lo}" cells.
   c. Apply this gate to all plots to maintain a consistent gate shape for all tubes of interest.
3. Use side scatter (a marker of cellular granularity) to characterize granulocyte precursor cells, monocyte precursor cells and primitive progenitor cells (Figure 4C).
   a. We label each cell subcategory based on ALDH activity and cellular complexity
   b. Granulocyte precursor cells correspond to ALDH^{hi}SSC^{hi} cells
   c. Monocyte precursor cells correspond to ALDH^{hi}SSC^{mid} cells
   d. Primitive progenitor cells (including hematopoietic and endothelial cells) correspond to ALDH^{hi}SSC^{low} cells
4. Within each group, fluorescence minus one (FMO) gates are used to examine specific surface marker expression within that cell population (Figure 4D).
   a. Axes should reflect the FSC and the fluorochrome of interest respectively. For example, FMO-PE axes should be FSC and PE for the Y and X axes.
   b. Apply this gate to all plots to maintain a consistent gate shape for all tubes of interest.
   c. Tip: you may choose to calculate the FMO of each fluorophore in a specific ALDH subcategory, on all cellular events, on all ALDH^{hi}, or all ALDH^{lo} cells depending on your specified analyses.
5. Following completion of gates for each fluorochrome, we can move to examining the full panels for analyses.
   a. First measure the frequency of cellular events using the gate from step 1.
   b. Measure the frequency of each fluorochrome on all cellular events, this will demonstrate the power of including elevated ALDH activity in your examination of progenitor cells.
   c. Next measure the frequency of each ALDH^{hi} subcategory if applicable to a specific cell type you are examining.
   d. Depending on your intended analyses, measure the co-expression of multiple surface markers within each ALDH^{hi} subcategory. For example, we frequently use the co-expression of CD34+/ CD133+ cells within the ALDH^{hi}SSC^{low} subcategory to characterize pro-vascular progenitor cells.
   e. Get creative with your analyses, look for different connections within each cell panel.

Please see (Hess et al., 2019; Terenzi et al., 2019) for other strategies specific to pro-vascular progenitor cell surface markers in peripheral blood.

LIMITATIONS

When conducting the protocol without SepMate tubes, it is critical not only to effectively layer the blood sample onto the Ficoll, but also to remove the buffy coat with precision. The use of SepMate tubes removes this limitation.

In addition, check published literature to examine the effectiveness of surface markers specific to your intended analyses. Some antibodies may bind more effectively to your cell of interest and therefore more applicable to your research. Additionally, more rare cells should be placed on brighter fluorophores to aid the examination of these cells in analyses.

TROUBLESHOOTING

Problem 1

There seems to be incomplete separation of whole blood into plasma, buffy coat, and red blood cell layers.

Potential solution

1. Ensure the whole blood is fresh (collected <24 h prior to beginning the experiment), if blood is not fresh, it may become coagulated and will not produce accurate results.
2. Reduce ejection speed of pipette aid and tilt SepMate tube (Figure 3A). This will help to slowly layer the whole blood onto the Ficoll.
3. Ensure the centrifuge is set to the appropriate configurations and balanced properly. If the centrifuge is not calibrated properly, separation will not be complete.

Problem 2

There is still a red ring surrounding my pellet following ammonium chloride addition (Figure 3D).

Potential solution

1. Add 9 mL of ammonium chloride and 1 mL of PBS+5% FBS to the sample tube and allow the solution to sit for 8 min. Next centrifuge the sample and repeat these steps until the pellet is opaque (Figure 3E).

Problem 3

I am unable to run the sample within the 24 h time limit and want to fix the cells in order to analyze them in the future, is this applicable?

Potential solution

1. While applicable to use fixed cells for the examination of cell surface markers by flow cytometry at a later date, the examination of ALDH expression requires live cells. Therefore, if researchers decide to fix the cells, they will not be able to examine ALDH expression in the sample.

Problem 4

DEAB does not seem to inhibit Aldefluor signal appropriately.

Potential solution

1. During the next experiment, ensure that the "ALDH-inhibited" tube is capped immediately following DEAB reagent addition. It is possible that the substrate is evaporating and is therefore unable to inhibit ALDH activity properly.

Problem 5

Aldefluor signal is dim or there is no clear separation of ALDH^{hi} cells

Potential solution

1. Consult Aldefluor kit brochure to confirm Aldefluor activation was completed properly. Furthermore, ensure proper mixing and incubation time prior to analyses. Activated Aldefluor should appear neon green immediately if it has been activated properly. Solution may appear cloudy, but this will not affect the potency of the Aldefluor. Please see https://cdn.stemcell.com/media/files/pis/29888-PIS_1_1_2.pdf.

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead and Technical Contacts, Daniella C. Terenzi (Daniella.terenzi@unityhealth.to) and [David A. Hess] (dhess@robarts.ca).

Materials availability
This study did not generate unique reagents.

Data and code availability
This study did not generate/analyze data sets or codes.

AUTHOR CONTRIBUTIONS
Conceptualization, D.C.T., S.V., and D.A.H; Methodology, D.C.T. and D.A.H.; Writing – Original Draft, D.C.T. and D.A.H; Writing – Review & Editing, all authors; Visualization, D.C.T., E.B., S.V., and D.A.H.; Funding Acquisition, S.V. and D.A.H.

DECLARATION OF INTERESTS
H.T. reports receiving honorarium from Boehringer Ingelheim and writing fees for unrelated diabetes-related manuscripts from Merck and Servier. S.V. holds a Tier 1 Canada Research Chair in Cardiovascular Surgery and reports receiving research grants and/or speaking honoraria from Amarin, Amgen, AstraZeneca, Bayer, Boehringer Ingelheim, Bristol-Myers Squibb, Eli Lilly, EOCI Pharmacomm Ltd, HLS Therapeutics, Janssen, Merck, Novartis, Novo Nordisk, PhaseBio, Sanofi, Sun Pharmaceuticals, and the Toronto Knowledge Translation Working Group. He is the President of the Canadian Medical and Surgical Knowledge Translation Research Group, a federally incorporated not-for-profit physician organization.

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