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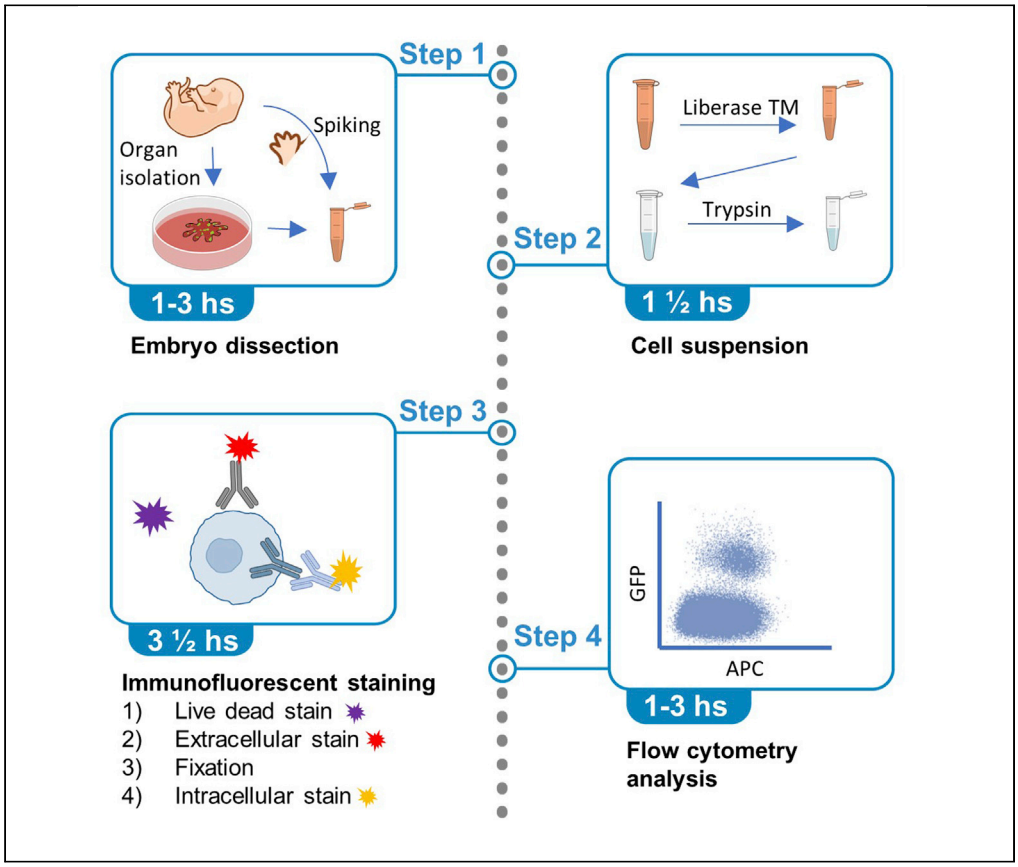
Flow cytometry detection of surface and intracellular antigens in pancreas from a single mouse embryo



We here report a flow-cytometry-based protocol to measure single-cell protein expression in small samples. The protocol is optimized for simultaneous detection of fluorescent proteins and intracellular and surface antigens in the embryonic pancreas from the mouse. Owing to low cell numbers, current protocols for flow cytometric analysis of embryonic tissues rely on tissue pooling. Our protocol enables analysis of one pancreas per sample, thereby facilitating detection of biological variation and minimizing the number of experimental animals needed.

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Highlights

Quantitative protein analysis of a single embryonic pancreas using flow cytometry

Simultaneous detection of reporter, cell surface, and intracellular proteins

Enables detection of biological variation and reduces the number of animals needed

Enables the study of heterogeneous cellular protein expression during organogenesis

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Flow cytometry detection of surface and intracellular antigens in pancreas from a single mouse embryo

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SUMMARY

We here report a flow-cytometry-based protocol to measure single-cell protein expression in small samples. The protocol is optimized for simultaneous detection of fluorescent proteins and intracellular and surface antigens in the embryonic pancreas from the mouse. Owing to low cell numbers, current protocols for flow cytometric analysis of embryonic tissues rely on tissue pooling. Our protocol enables analysis of one pancreas per sample, thereby facilitating detection of biological variation and minimizing the number of experimental animals needed.

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

BEFORE YOU BEGIN

As the protocol is essentially a very long one-day experiment with no pause points, it is essential that all reagents are prepared in advance, and that mouse breeding is carefully timed to match the booking of the flow cytometry equipment.

DETERMINE OPTIMAL WORKING DILUTION OF YOUR ANTIBODY PANEL

Timing: Variable from 1 – 7 days

1. Identify and order primary and (if applicable) secondary antibodies suitable for your application
   a. Preferably use antibodies that have been verified for flow cytometry by either your own group, the vendor or another research group. To find verified antibodies we recommend the use of an online search engine such as BenchSci (https://www.benchsci.com/products/ai-assisted-antibody-selection)
   b. Consider the technical specifications of your flow cytometer when determining the ideal combination of fluorophores. Keep in mind compatibility with the emission and excitation spectra of any fluorescent proteins expressed by the cells in your sample (see Table 1 for an example). The use of a fluorescence spectra analyzer is recommended (https://www.biolegend.com/en-us/spectra-analyzer).
2. Determine optimal dilution factor of your antibody panel if not already known from previous experiments

Table 1. Fluorophores and fluorescent proteins used in example data set

Fluorophore/ fluorescent protein	CFP	GFP	Cy3	APC	Ghost dye Red-780
Emission peak (nm)	480	510	568	660	780
Excitation laser (nm)	350/405	488	488/532	594/ 633	633
Marker name	E-CADHERIN	PDX1	P120CTN	CD133	Viability
Marker location	Cytosol	Cytosol	Intracellular	Extracellular	Intracellular and extracellular
Fluorophore format	Reporter mouse	Reporter mouse	Unconjugated primary + fluorophore conjugated secondary	Fluorophore conjugated antibody	Dye

The name and location of the protein marked by each fluorophore is given above, as well as the experimental format of marking.

a. Test the vendor or scientific literature based recommended dilution factor and at least a 2× more diluted and a 2× more concentrated solution (Example: if recommendation is 1:200, test 1:100, 1:200 and 1:400) on your cells of interest following the entire protocol outlined below.

Setup mouse mating for embryonic tissue

Timing: 2–3 days (but needs to be set up several days in advance depending on embryonic stage needed)

3. Setup mating of mice (see alternatives below)
   a. Move one male age 6 weeks to 8 months to each of 3–4 clean breeding cages (if time permits this can be done the previous evening)
   b. Add two females age 6–12 weeks to each breeding cage in the afternoon
4. Check for vaginal plug the next morning
   a. Separate plugged females to a clean cage, and record number of plugged females
   b. Separate males and unplugged females
   c. Day of vaginal plug is recorded as embryonic day 0.5 (E0.5)
   d. Carefully record mouse ID and genotype of both male and female along with day of setup and day of plug (see Table 2 for an example)
5. Wait the required amount of days according to which embryonic stage you aim to analyze. I.e. for E12.5 tissue you need to set up breeding 14 days prior to the day you plan to perform the flow cytometry experiment (see Figure 1)

Note: Make sure you have the necessary ethical and breeding permits for laboratory animal use from your local authorities

Alternatives: Mouse strains used can be any wild type strain or a tissue or cell specific reporter suitable for cell sorting. In our exemplary experiment (see Table 1 and 2) we have used the pancreas specific Pdx1-GFP reporter (Holland et al., 2006) and the epithelial cell specific Ecad-CFP reporter (Snippert et al., 2010). When designing your breeding setup, consider the need for single reporter positive embryos for controls (see Table 3). In our example we set up Ecad-CFP homozygous or heterozygous female mice with Pdx1-GFP heterozygous male mice (see Table 2).

Table 2. Example of table for record keeping of mating between one male and two females

Cage #	Male ID	Male genotype	Female ID	Female genotype	Date setup	Date plugged	Date dissected (Embryonic stage)
PN-EXP206	9943	Pdx1-GFP heterozygous	432	Ecad-CFP homozygous	18/5/2019	21/5-2019	1/6-2019 (E11.5)
PN-EXP206	9943	Pdx1-GFP heterozygous	434	Ecad-CFP heterozygous	18/5/2019	21/5-2019	1/6-2019 (E11.5)

Book flow cytometry equipment

Timing: 10 min

6. As flow cytometry equipment is often a shared resource and sometimes require technical assistance, make sure to book equipment and technical staff well in advance
   a. As a rule of thumb, the equipment should be booked for 4 PM–6 PM on the day of the dissection of the embryonic tissue if the protocol is started at 8 AM.

Note: Carefully consider the choice of cytometer and make sure the fluorophores and/or fluorescent proteins match the cytometer configuration. The exemplary protocol includes CFP, GFP, Cy3, APC and Red-780 (see Table 1). The protocol can be optimized to fit the type of cytometer available to the end user by using fewer or different fluorescent dyes

Prepare reagents for tissue lysis and immunostaining

Timing: 2 h

7. Prepare Liberase TM aliquots
   a. Resuspend at a concentration of 22.6 mg/mL by adding the appropriate amount of ddH_2O to the tube (221 µL to 5 mg) and pipetting slowly up and down.
   b. Aliquot 10 µL Liberase TM into each PCR tube and freeze at −20°C
8. Aliquot 40 µLDNAse I (10U/µL) into Eppendorf tubes and freeze at −20°C
9. Aliquot heat inactivated FBS into sterile 50 mL tubes with 33,3 mL/tube and freeze at −20°C
10. Aliquot 0.25% Trypsin-EDTA into Eppendorf tubes with 1 mL/tube and freeze at −20°C
11. Prepare buffered PBS (without calcium and magnesium) with 1 mM EDTA 4Na 2H2O:
    a. Weigh 0.186 g EDTA 4Na 2H2O and dissolve in 500 mL sterile PBS without calcium and magnesium.
    b. Store at 20°C–22°C for an extended time.
12. Prepare 2% formaldehyde solution from paraformaldehyde (PFA) while working in a chemical safety cabinet
    a. Add 4 g of paraformaldehyde to 196 mL sterile PBS in a 250 mL beaker
    b. Heat at 70°C–80°C for ca. 1 h until all powder has dissolved. Monitor temperature closely, and never bring the solution above 80°C
    c. Cool down to 20°C–22°C
    d. Aliquot into 15 mL tubes with 10 mL/tube and freeze and store for up to one year at −20°C, unless used immediately

⚠ CRITICAL: Paraformaldehyde is a toxic chemical, which targets the respiratory system and should be used according to the safety instructions. Use gloves and work in a chemical safety cabinet, or use eye/face shield and respirator cartridge type N100 (US), type P1 (EN143) respirator filter, type P3 (EN 143) respirator cartridges. Always use freshly thawed formaldehyde, as it polymerizes when exposed to oxygen, which will compromise the cross-linking efficiency.

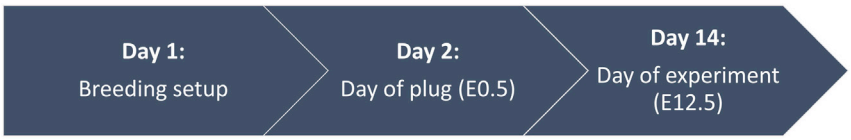

Figure 1. Example of an experimental timeline for analysis of E12.5 embryos from breeding setup to flow analysis

Alternatives: Commercially available ampules of premade aqueous solution of 2% formaldehyde without additives can replace the freshly made formaldehyde. Commercially available concentrated formaldehyde aqueous solutions not in ampules should be avoided, as they often include stabilizing agents such as methanol. (Fox et al., 1985; Helander, 2000)

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat monoclonal anti-CD133 (Prominin) APC conjugated (clone13A4) (FACS 1:100)	eBioscience/Invitrogen	Cat#: 17-1331-81 RRID: AB_823120
Mouse polyclonal anti-p120ctn (pp120) (FACS 1:1000)	BD Transduction Labs	Cat#: 610134 RRID: AB_397537
Cy™3 AffiniPure Goat Anti-Mouse IgG, Fcγ subclass 1 specific, polyclonal antibody	Jackson ImmunoResearch	Cat#: 115-165-205 RRID: AB_2338694
Ghost Dye Red-780	Tonbo Biosciences	13-0865-T100 RRID: N/A
Chemicals, peptides, and recombinant proteins		
DMEM/F12 medium	Thermo Fisher Scientific	Cat#: 11320033 RRID: N/A
Sterile PBS with Mg/Ca	Gibco	Cat#: 14040083 RRID: N/A
Sterile PBS without Mg/Ca	Gibco	Cat#: 14190094 RRID: N/A
Paraformaldehyde powder	VWR	Cat#: 28794.295 RRID: N/A
DNAse I, RNase free (1U/µL)	Thermo Scientific	Cat#: EN0521 RRID: N/A
Liberase™ TM Research Grade	Roche	Cat#: 5401119001 RRID: N/A
Fetal Bovine Serum (FBS)	Gibco	Cat#: 26140079 RRID: N/A
EDTA, Disodium Salt Dihydrate, Molecular Biology Grade (MW 372.24)	Merck	CAS#: 6381-92-6 RRID: N/A
0.25% Trypsin-EDTA with phenol red	Thermo Fisher Scientific/Gibco	Cat#: 25200072 RRID: N/A
Experimental models: Organisms/strains		
Mouse: C57BL/6BomTac	Taconic	Cat#: B6JBOM-F/M RRID: N/A
Mouse: Pdx1-GFP: Pdx1tm1Egs	Holland et al. 2006	RRID: MGI:3695580
Mouse: Ecad-CFP: Cdh1tm1Cle	Snippert et al., 2010	RRID: IMSR_JAX:016933
Software and algorithms		
FCS Express™	De Novo Software https://denovosoftware.com/	N/A
Other		
Dumont #5 Forceps in Inox-Biology	Fine Science Tools	Cat#: #5SF RRID: N/A
Cell strainer for flow cytometry such as Filcons S sterile orange cup 30 µm	BD Biosciences/BD™ Medimachine	Cat#: 340625 RRID: N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Flow cytometer: BD FACS Aria III sorter or other Website: https://www.bdbiosciences.com/en-eu/instruments/research-instruments/research-cell-sorters/facsaria-iii	BD Biosciences	N/A
Cytometer Setup & Tracking beads (depends on cytometer used)	BD Biosciences	Cat# 655050 RRID: N/A
Stereoscope for embryo dissection	Nikon	Cat# SMZ1270 RRID: N/A
Optional: Stereoscope equipped for fluorescence imaging	N/A	N/A
Water bath	N/A	N/A
Thermomixer which can heat up to 37°C and vibrate at 950 rpm	N/A	N/A
Micropipettes	N/A	N/A
Cooled centrifuge (4°C) which can hold 1.5 mL eppendorf tubes and run at 500 *g*	N/A	N/A
3D gentle rotator/shaker or similar for antibody incubation	N/A	N/A

MATERIALS AND EQUIPMENT

The flow cytometer used in this study was a BD FACS Aria III sorter (BD Biosciences) equipped with 375 nm/405 nm, 445 nm/488 nm, 561 nm, and 633 nm laser lines. For this experiment, a 100 μm nozzle /20 psi configuration was used together with the 375 nm, 445 nm, 561 nm and 633 nm lasers. We chose this sorter because we needed laser lines able to detect CFP, GFP, Cy3, APC and Ghost Dye RED-780 (see also Table 1).

Alternatives: Depending on the need for cell sorting and fluorescent spectra, different cytometers can be used. The protocol can be optimized to fit the type of cytometer available to the end user by using fewer or different fluorescent dyes.

STEP-BY-STEP METHOD DETAILS

Prepare reagents

Timing: 30 min

This step prepares the buffers and reagents, which are made on the morning of the experiment.

Alternatives: Any left over buffers can be re-used for a later experiment, as long as they are stored as stated below.

1. Make 10 mL DMEM/F12 medium with 10% FBS working under sterile conditions in a flow bench:
   a. Thaw FBS in a waterbath
   b. Add 1 mL FBS to 9 mL DMEM/F12 medium in a sterile 15 mL tube and gently mix by inverting the tube
   c. Store at 4°C and use within one week
2. Make 10 mL 0.0125% Trypsin-EDTA:
   a. Thaw one vial of 0.25% Trypsin-EDTA at 20°C–22°C
   b. Add 0.5 mL 0.25% Trypsin-EDTA to 9.5 mL PBS w EDTA in a 15 mL tube
   c. Store at 4°C and use within one week
3. Make 1 L 10% FBS in PBS (henceforth FBS/PBS):
   a. Thaw 3 vials of FBS in a waterbath

b. Add 100 mL of FBS to 900 mL of sterile PBS
c. Store at 4°C and use within two weeks

4. Make 500 mL of 10% FBS in PBS with 0.5% Tween-20:
   a. Take 497.5 mL FBS/PBS from step 3 and add 2.5 mL Tween-20
   b. Store at 4°C and use within two weeks

Pause point: Maximum of one week. As these buffers and reagents can be stored for one to two weeks, it is possible to prepare them slightly in advance, for instance the previous day.

5. Thaw one vial of 2% formaldehyde (100 µL needed per sample) and store on wet ice for use in fixation (step 17).

Dissect embryonic tissue for analysis

Timing: 1–3 h depending on number of plugged female mice

Table 3. Example of control samples and samples collected for an experiment with two reporter mouse strains: Ecad-CFP (Snippert et al., 2010) and Pdx1-GFP (Holland et al., 2006)

Name	Embryo genotype	Tissue	Purpose
No fluorophore control	CFP Reporter negative, GFP reporter negative	Pancreas and paws (if no reporter negative embryos are identified, paw tissue can be used exclusively if paws do not express the reporter)	To control for autofluorescence and set up gating (at least one)
Secondary antibody only control	CFP Reporter negative, GFP reporter negative	Pancreas and paws (if no reporter negative embryos are identified, paw tissue can be used exclusively if paws do not express the reporter)	To control for unspecific secondary antibody binding and set up gating (at least one)
Ghostdye only control	CFP Reporter negative, GFP reporter negative	Pancreas and paws (if no reporter negative embryos are identified, paw tissue can be used exclusively if paws do not express the reporter)	For compensation and gating (at least one for first experiment)
Cy3 only control	CFP Reporter negative, GFP reporter negative	Pancreas and paws (if no reporter negative embryos are identified, paw tissue can be used exclusively if paws do not express the reporter)	For compensation and gating (at least one for first experiment)
APC only control	CFP Reporter negative, GFP reporter negative	Pancreas and paws (if no reporter negative embryos are identified, paw tissue can be used exclusively if paws do not express the reporter)	For compensation and gating (at least one for first experiment)
GFP only control	CFP Reporter negative, GFP reporter positive	Pancreas and paws	For compensation and gating (at least one for first experiment)
CFP only control	CFP Reporter positive, GFP reporter negative	Pancreas and paws	For compensation and gating (at least one for first experiment)
Sample 1,2,3…	CFP Reporter positive, GFP reporter positive	Pancreas and paws	Samples for measurements (at least 3)

In this step, embryonic tissue is dissected for the analysis.

Alternatives: The protocol was optimized for embryonic pancreas, but can be used for other embryonic inner organs (lungs, liver, thyroid, etc).

Note: The timing depends on the number of embryos dissected and the experience of the researcher. One pregnant mouse with 10 embryos routinely takes us 40 min to dissect, and should take no longer than 1 h to prevent cell death. See Problem 3: High cell death. To allow time for the rest of the experiment, the total time for dissection should not exceed 3 h. In our experience, we can process maximum 3 pregnant mice per experiment. If possible, work 2 persons on this part of the protocol to ensure a steady pace.

6. Prepare a stereoscope laboratory station with wet-ice bucket, two dissection forceps and ice-cold sterile 1× PBS
7. Prepare 1.5 mL Eppendorf tubes with 500 µL DMEM medium in each and place on wet ice
8. Euthanize the pregnant female by cervical dislocation

9. Isolate your organ of interest from embryos
   a. Dissect the uterus containing the embryos from the pregnant female
   b. Move embryos to ice-cold sterile 1× PBS in a Petri dish stored on wet ice
   c. Dissect the embryos one by one from the uterus and move embryos to a small clean Petri dish containing ice-cold sterile 1× PBS
   d. Assign embryos ID numbers starting from 1

   Note: The sex of the embryos were not determined

   Optional: Check for fluorescence: If using fluorescent reporter mice, the expression of fluorescence can be checked under a stereoscope equipped for fluorescence imaging. Any non-fluorescent embryos should be used for control samples (see Table 3). Note down the ID number of fluorescent positive and negative embryos
   e. Under a stereoscope, dissect your organ of interest from the embryos using two #1 Dumont forceps

   Alternatives: Other fine forceps or needles can also be used for dissection
   f. Dissect the four paws from each embryo

   Optional: Save tissue for genotyping: When using a reporter mouse, one paw from each embryo can be transferred to a PCR tube on wet ice for later verification of genotype by PCR (not included in this protocol)

10. Move the organ of interest and one paw (E14.5) or all remaining paws (E11.5) from the same embryo into ice-cold 500 µL DMEM in Eppendorf tubes on wet ice.

⚠ CRITICAL: Make sure your organs of interest are all dissected in a similar manner, taking care not to include any nearby tissue, which could greatly affect your sample.

⚠ CRITICAL: The "spiking" of the sample with paw tissue minimizes loss of cells of interest during the processing steps, enabling the analysis of very small tissue samples. The paws are used for spiking, as they did not express any of our antigens of interest. Always check that this is also the case for your choice of antigens, and adjust the protocol accordingly to include an alternative spiking tissue. For our smallest (E11.5) samples we added additional spiking cells from paws (up to a total of 6) of any surplus embryos with a genotype we did not need to analyse.

Preparation of cell suspension

Timing: 1 h, 40 min

This step disaggregates the tissues and prepares a single cell suspension ready for immunofluorescent staining. The digestion step is performed essentially as describe in (Jørgensen et al., 2018).

11. Digest tissue with Liberase TM
    a. To sample in DMEM, add 1.25 μL Liberase TM (22.6 mg/mL)
    b. Add 4 μL DNAse I (10U/μL)
    c. Incubate at 37°C, 20 min, at 950 rpm (rounds per minute) on a thermomixer.

⚠ CRITICAL: Triturate gently every 3–4 min using a P1000 pipette. Make sure you change tips between samples

    d. Centrifuge tubes at 500 *g* for 4 min at 4°C and carefully aspirate and discard the supernatant (Henceforth: Spin).

12. Digest tissue with Trypsin
    a. Resuspend in 100 μL of 0.0125% Trypsin-EDTA with 4 μL DNAse I (10U/ μL) added
    b. Incubate 37°C for 10 min, at 950 rpm on thermomixer

⚠ CRITICAL: Triturate gently every 3–4 min using a P1000 pipette. Make sure you change tips between samples

    c. Check that tissue has completely disintegrated
    d. Spin

13. Resuspend pellet with 1000 μL ice-cold 10% FBS in DMEM to inactivate the trypsin
14. Filter the cells into Eppendorf tubes using single-use cell strainers to make a single cell suspension. Wash the strainer with 500 μL FBS/PBS into tube.

⚠ CRITICAL: It is important that all tissue be digested during this step. Incomplete digestion of tissue in step 12) will result in visible clumps of cells or tissue and will greatly affect downstream processing. See Problem 1: Incomplete digestion.

Immunofluorescent staining of cells

Timing: 3 h, 30 min

This step labels the cells using immunofluorescent staining for dead cells and for extracellular and intracellular antigens. Staining of dead cells is performed first, followed by extracellular staining, then cell fixation and finally intracellular staining. Table 4 shows an overview of the exemplary staining protocol.

⚠ CRITICAL: It is important that care is taken not to lose cells during the washing steps (spin, remove supernatant, resuspend). See Problem 2: Low cell yields.

⚠ CRITICAL: Keep all reagents on wet-ice. See also Problem 3: High cell death

Note: Blocking: 10% FBS is included in all steps below, except Ghostdye staining and fixation, to block unspecific protein binding during staining and keep cells dissociated and alive (before fixation). There is no Fc receptor blocking step, because we analyse cells from embryonic internal organs, which do not to our knowledge contain Fc receptors. For users who wish to test our protocol on cell types which do express Fc receptors (mainly immune system cells), such a step should be included (Hulspas et al., 2009). See also Problem 5: High noise/background

Table 4. Exemplary staining protocol with processing steps for all controls and samples

Name	Viability stain (step 15)	Primary antibody for extracellular antigen (step 16)	Fixation (step 17)	Primary antibody for intracellular antigen (step 18)	Secondary antibody for intracellular antigen (step 19)
No fluorophore control	-	-	2% PFA	-	-
Secondary antibody only control	-	-	2% PFA	-	Cy3 Goat Anti-Mouse IgG, Fcγ subclass 1 specific, polyclonal antibody (1:1000)
Ghostdye only control	Ghost dye Red-780 (1:1000)	-	2% PFA	-	-
Cy3 only control	-	-	2% PFA	Mouse polyclonal anti-P120CTN (1:1000)	Cy3 Goat Anti-Mouse IgG, Fcγ subclass 1 specific, polyclonal antibody (1:1000)
APC only control	-	Rat monoclonal anti-CD133 APC-conjugated (1:100)	2% PFA	-	-
GFP only control	-	-	2% PFA	-	-
CFP only control	-	-	2% PFA	-	-
Sample 1,2,3…	Ghost dye Red-780 (1:1000)	Rat monoclonal anti-CD133 APC-conjugated (1:100)	2% PFA	Mouse polyclonal anti-P120CTN (1:1000)	Cy3 Goat Anti-Mouse IgG, Fcγ subclass 1 specific, polyclonal antibody (1:1000)

Note: Controls: See Table 3 for an overview of controls used in our exemplary experiment. These are the absolute minimum of controls needed for a multicolor labeling experiment. For a discussion of other types of recomended controls see (Hulspas et al., 2009; Maecker and Trotter, 2006). Isotype controls are sometimes also used to detect unspecific binding of antibody. These are antibodies of the same isotype of immunoglobulin as the specific antibody, but are either myeloma derived antibodies of unknown specificity, or are raised against an antigen that is not epressed by the cells. We did not use isotype controls, for the reasons described by (Hulspas et al., 2009; Maecker and Trotter, 2006) and discussed in Problem 5: High noise/background.

15. Stain with Ghostdye for detection of dead cells
    a. Spin
    b. Remove supernatant and resuspend in 1000 µL pure sterile PBS.

    Note: FBS is removed by washing with pure sterile PBS before staining with Ghost dye to optimize the staining efficiency as FBS will react with and bind up some proportion of the Ghost dye. If your cells do no tolerate a FBS-free environment, a low concentration of FBS can be used, but the concentration of the Ghost dye should be optimized accordingly in the same concentration of FBS.
    c. Spin

d. Resuspend in 1000 μL pure sterile PBS
e. Spin Ghostdye only control and samples. The remaining controls which are not stained with Ghostdye (see Tables 3 and 4) are set aside (in PBS) on wet ice during steps e-i
f. Meanwhile prepare 100 μL of dPBS with 1:1000 dilution of Ghost dye (Ghost dye Red-780) per sample.
g. Resuspend cells in Ghost dye solution
h. Mix immediately
i. Incubate 30 min at 4°C (in cold room or refrigerator) protected from light
j. Spin all samples and controls
k. Resuspend in 1000 μL FBS/PBS
l. Spin
m. Resuspend in 1000 μL FBS/PBS

16. Stain with primary antibody for extracellular antigen
    a. Set aside controls which do not require staining with primary antibody for extracellular antigen (see Tables 3 and 4) in FBS/PBS on wet ice
    b. Prepare enough primary antibody dilution to stain all your samples, allowing 100 μL per sample. Note that extra diluted antibody should be made to account for pipetting error (at least 10% extra volume). Prepare primary antibody dilution in FBS/PBS. For CD-133-APC we dilute 1:100: For a total of 1 mL (enough for 9 samples) add 10 μL antibody in 990 μL FBS/PBS and mix gently. For alternative antibody panels the optimal dilution factor needs to be determined by end user (see first step in the "before you begin" section).
    c. Spin samples and controls requiring staining with primary antibody for extracellular antigen (see Table 3)
    d. Resuspend cells in 100 uL diluted primary antibody per sample
    e. Incubate for 30 min rotating at 4°C protected from light. Use a 3D gentle rotator/shaker placed at 4°C and cover tubes in foil.
    f. Add 1000 μL ice-cold 10% FBS/PBS
    g. Spin all samples and controls (including controls set aside in step 16a)
    h. Resuspend in 1000 μL ice-cold pure sterile PBS (to remove FBS before fixation)

17. Fixation

    Note: All samples and controls should be included in the fixation step
    a. Spin
    b. Resuspend in 100 μL 2% PFA (freshly thawed) and leave tubes at 20°C–22°C for 15 min protected from light.
    c. Add 1000 μL ice-cold pure sterile PBS
    d. Spin
    e. Resuspend in 1000 μL ice-cold FBS/PBS

18. Stain with primary antibody for intracellular antigen
    a. Spin all samples and controls
    b. Meanwhile prepare enough primary antibody dilution to stain all your samples. Prepare in 100 μL FBS/PBS with 0,5% Tween20 per sample. For mouse-anti-P120CTN we use a 1:1000 dilution: For a total of 1 mL (enough for 9 samples) add 1 μL antibody in 999 μL FBS/PBS with 0,5% Tween20 and mix gently. For alternative antibody panels the optimal dilution factor needs to be determined by end user.
    c. Resuspend cells from samples and controls requiring primary antibody for intracellular antigen (see Table 3) in 100 uL diluted primary antibody per sample. All other controls are resuspended in 100 μL FBS/PBS with 0,5% Tween20.
    d. Incubate for 30 min rotating at 4°C protected from light. Use a 3D gentle rotator/shaker placed at 4°C and cover tubes in foil.

e. Add 1000 μL FBS/PBS with 0,5% Tween20.
f. Spin
g. Resuspend in 1000 μL FBS/PBS
h. Repeat this wash at least ×1

19. Stain with secondary antibody for unconjugated primary
    a. Spin all samples and controls
    b. Meanwhile prepare enough secondary antibody dilution to stain all your samples and some controls (see Tables 3 and 4). Prepare in 100 μL FBS/PBS per sample. For the Cy3 Goat Anti-Mouse secondary antibody we use a 1:1000 dilution: For a total of 1 mL (enough for 9 samples) add 1 μL antibody in 999 μL FBS/PBS with 0,5% Tween20 and mix gently. For alternative antibody panels the optimal dilution factor needs to be determined by end user.
    c. Resuspend cells from samples and controls requiring secondary antibody for intracellular antigen (see Tables 3 and 4) in 100 μL of diluted secondary antibody per sample. All other controls are resuspended in 100 μL FBS/PBS with 0,5% Tween20. Incubate for 10 min rotating at 4°C protected from light. Use a 3D gentle rotator/shaker placed at 4°C and cover tubes in foil.
    d. Add 1000 μL FBS/PBS and spin
    e. Repeat wash at least ×2.
20. Prepare samples for flow analysis
    a. Spin all samples and controls
    b. Resuspend in 100 μL ice-cold 10% FBS/PBS.
    c. Immediately analyze samples using flow cytometry (ready ca 16.30 PM).

⚠ CRITICAL: It is important that samples are analyzed immediately, or they may lose fluorescence intensity. See Problem 4: Low fluorescence

Flow cytometry analysis of cells

Timing: 1–3 h depending on number of samples and cells

In this step, the cells are gated based on their light-scattering properties and fluorescent intensities from endogenous fluorescence (reporter) and from immunofluorescent staining, followed by analysis and optional sorting.

Note: In the following, we will outline the gating strategy, but exclude any technical information about operating the equipment as this is highly instrument specific and should be done according to the manufactures instruction for the particular instrument.

Note: Optimally, the preparation and calibration of the flow cytometer as described in step 21 below should be done during the staining procedure described above, so the stained cells can be analyzed immediately.

⚠ CRITICAL: Performing compensation to correct for fluorescent spectra overlap is critical when using multiple fluorphores, especially if they have overlapping emission spectra, as for instance CFP and GFP used in the exemplary data (Maecker and Trotter, 2006). The need for compensation could also be dependent on cytometer configuration. We used the single fluorphore/fluorescent protein controls described in Table 3 to perform compensation in step 22

21. Prepare and calibrate the flow cytometer according to manufacturer's instructions
    a. Startup system and select appropriate laser lines and filters
    b. Perform fluidics start-up and de-bubbling
    c. Calibrate laser delays
    d. If sorting, calibrate drop delay and side streams
    e. Setup experiment or load previous settings

Table 5. Average cell yield per sample

Embryonic age	Total cell yield/ sample		Total live cells/ sample			GFP positive pancreatic epithelial cells/ sample		
-	Range	Mean	Range	Mean	Mean % of all cells	Range	Mean	Mean % of total live cells
E11.5	9585-424231	144128	13618-422761	143637	99,54	15-948	384	0,33
E14.5	5338-132055	56539	5303-131325	56175	99,3	5-5839	2613	3,87

i. You will need plots of side scatter (SSC-A) versus forward scatter (FSC-A), FSC-H versus FSC-W, Ghost dye versus FSC-A, and your chosen fluorophores versus each other (reporter or immunostaining based). See examples in Figures 3, 4, and 5.

f. Adjust and save gains and voltages as appropriate for the cytometer and fluorophore panel

22. Acquire and save single fluorophore/fluorescent protein controls for compensation to correct for spectra overlap
    a. Use single fluorphore/fluorescent protein controls (examples described in Table 3) to perform compensation according to manufactures' instructions (automatic or manual).
23. Set gates for your fluorophores based on the controls (see Figures 3, 4, and 5 for examples)
    a. Use the no fluorophore control to set gate 1 on the FSC-A vs SSC-A plot (Figure 3A) to remove debris
    b. Use the no fluorophore control to set gate 2 on the FSC-W vs FSC-H (Figure 3B) to remove doublets
    c. Use the Ghost dye only control to set gate 3 and remove dead cells (See result of this gating on a stained sample in Figure 4C)

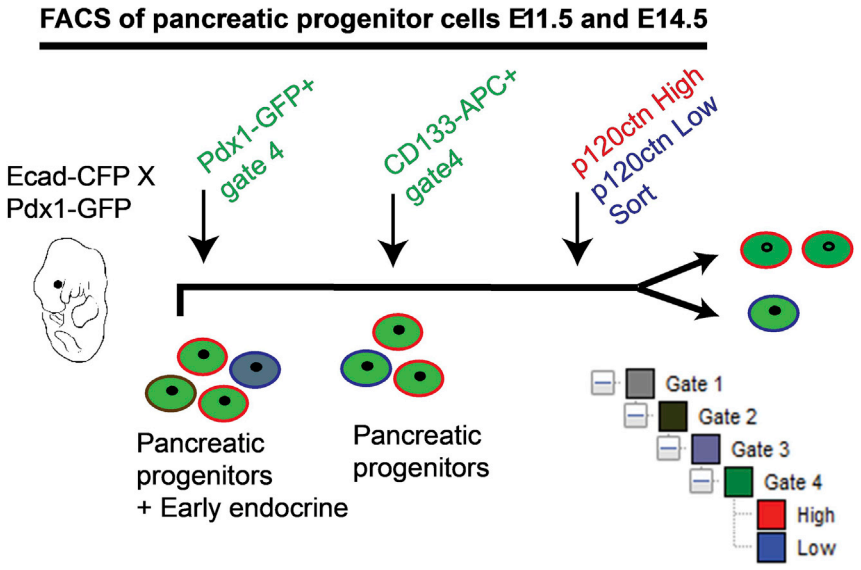

Figure 2. Example of an experimental strategy for flow cytometry analysis of Pdx1-GFP+ and CD133-APC+ pancreatic progenitors and sorting into populations with high versus low P120CTN expression

Pdx1-GFP was used as a marker for pancreatic epithelial cells, while CD133-APC was used to exclude CD133 negative early endocrine cells. Figure reprinted in with permission from (Nyeng et al., 2019).

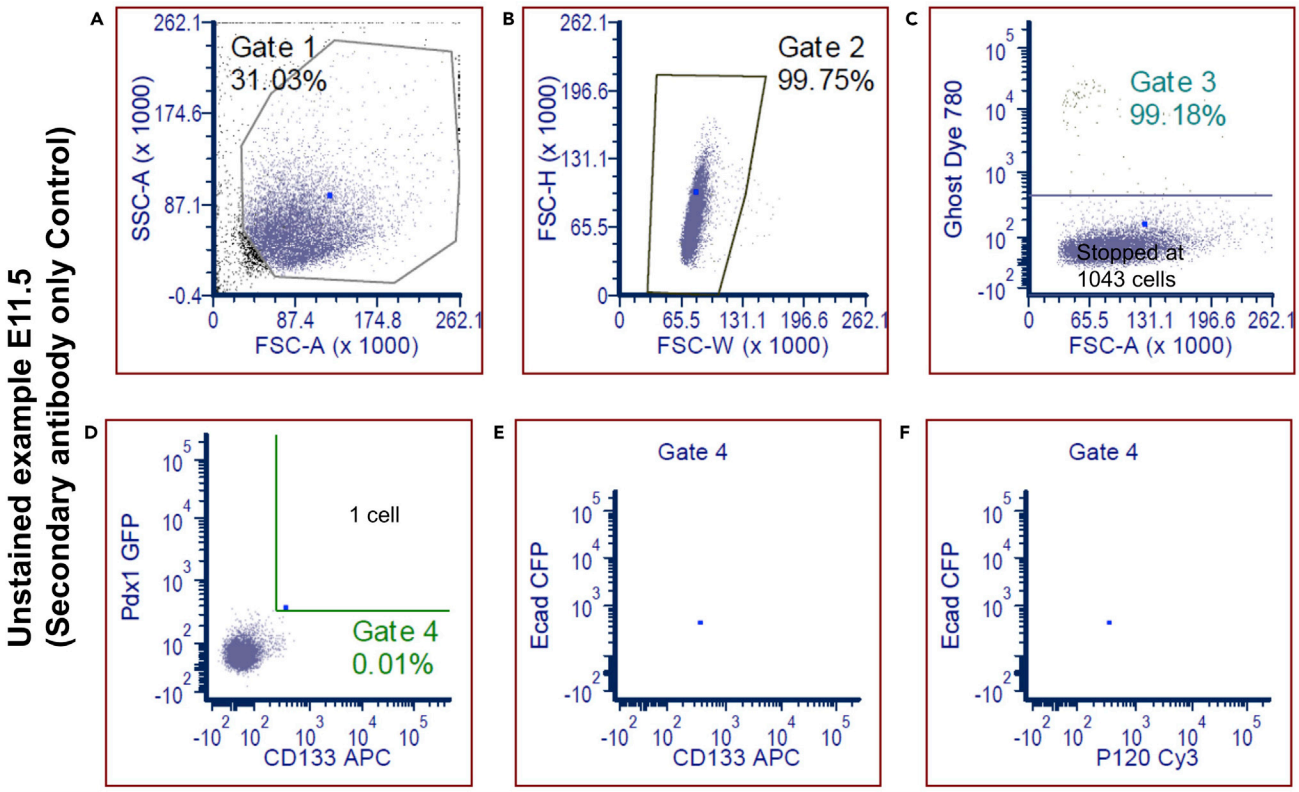

Figure 3. Data example of secondary antibody only control for E11.5 pancreas

The gating strategy is shown. Acquisition was stopped at 1043 cells. Gate 1 (A) identifies the cells, gate 2 (B) excludes doublets and clumbs, gate 3 (C) excludes dead cells and gate 4 (D–F) identifies the pancreatic progenitor cells. In this case, only one cell is identified (background), as reporter negative cells were used for this control. Figure reprinted with permission from (Nyeng et al., 2019).

d. Use the no fluorophore control, the secondary antibody only control and the single fluorophore/fluorescent protein controls to set gate 4 (see Figure 3D and result of this gating on a stained sample in Figure 4D)
   i. The no fluorophore control should have no positive cells in gate 4
   ii. The secondary antibody only control should have no positive cells in gate 4
   iii. The Cy3 only, APC only, CFP only and GFP only controls should reflect their relative genotypes or staining

Note: FMO controls (controls that include all fluorophores except one) were not included in the exemplary experiment, but could be useful for gating in a first pilot study with a new antibody panel. See (Maecker and Trotter, 2006) for more details.

Optional: If you wish to sort your cells, this needs to be set up with sorting gates (see example in Figure 4 and 5 where red and blue cells are sorted into two fractions based on bimodal distribution of P120CTN expression).

24. Record (and optionally sort) the samples one at a time. Record all the events in each sample.

EXPECTED OUTCOMES

From a successful E11.5 pancreas analysis you should be able to analyze/sort on average 300–900 GFP positive (*Pdx1* expressing) pancreatic epithelial cells, and from one E14.5 pancreas on average 2000–5000 GFP positive cells (see Table 5). The low fraction of GFP positive cells out of total cells is

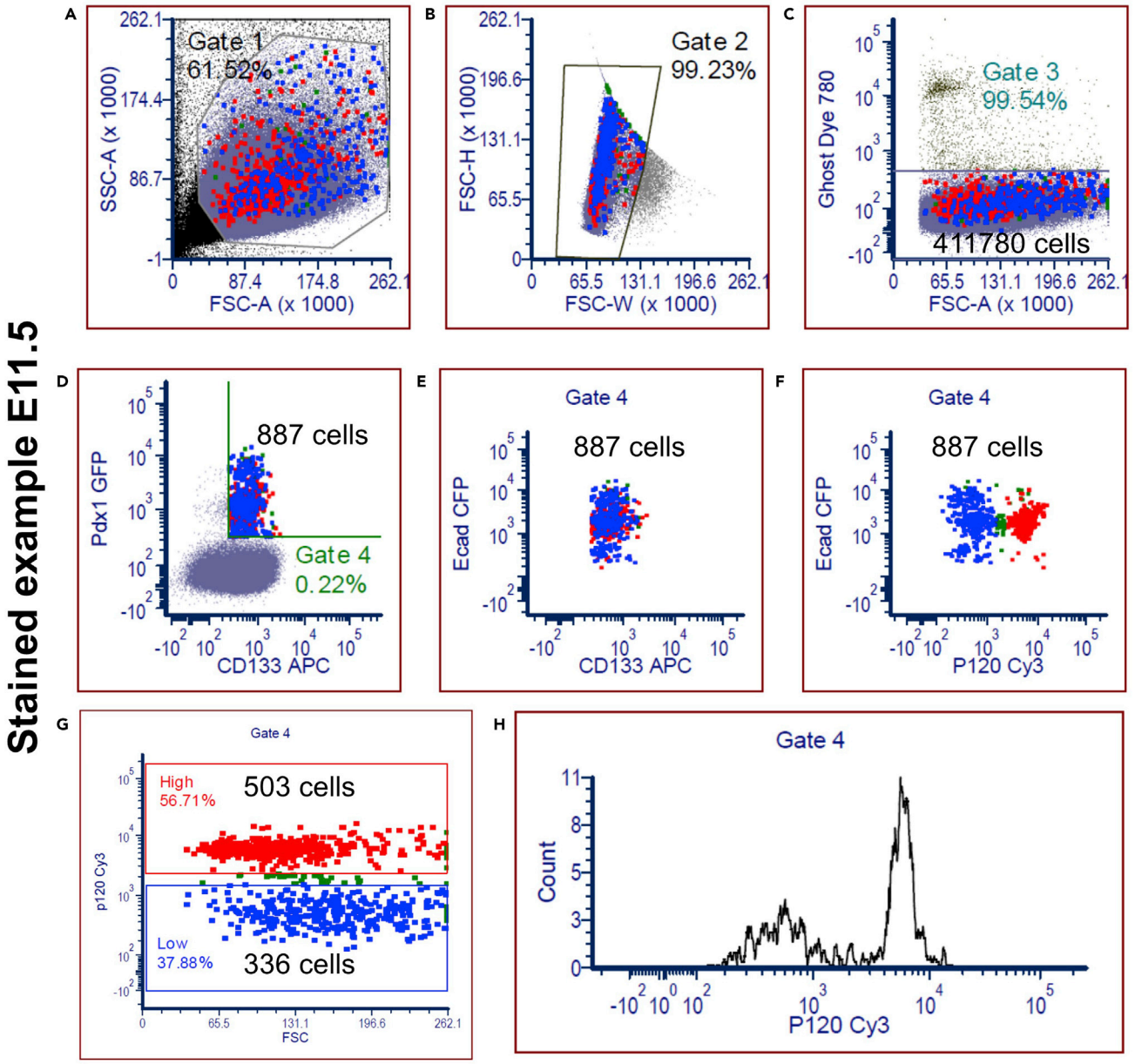

Figure 4. Representative data example of sample from a single E11.5 pancreas

The gating strategy, cell sorting and intensity diagram for P120CTN is included. Gate 1 (A) identifies the cells, gate 2 (B) excludes doublets and clumbs, gate 3 (C) excludes dead cells and gate 4 (D–G) identifies the pancreatic progenitor cells (887 cells). A clearly distinguishable GFP+, APC+ population is seen in D. Sorting in P120CTN high and low cells is shown in G, while the P120CTN expression profile is shown in H. Figure reprinted with permission from (Nyeng et al., 2019).

due to the addition of non-pancreatic cells to the sample. Please note that the higher number of cells used for spiking the E11.5 samples relative to the E14.5 samples is reflected in a higher number of total cells, but not GFP cells, in the E11.5 samples. In all succesful experiments, over 99% of the analyzed single cells were alive based on Ghost dye staining.

We used the described technique to analyze the content of the adhesion junction proteins E-CADHERIN and P120CTN in pancreatic epithelial cells. See Figure 2 for experimental design and gating strategy. Cells from Ecad-CFP and Pdx1-GFP double positive embryos were stained for the

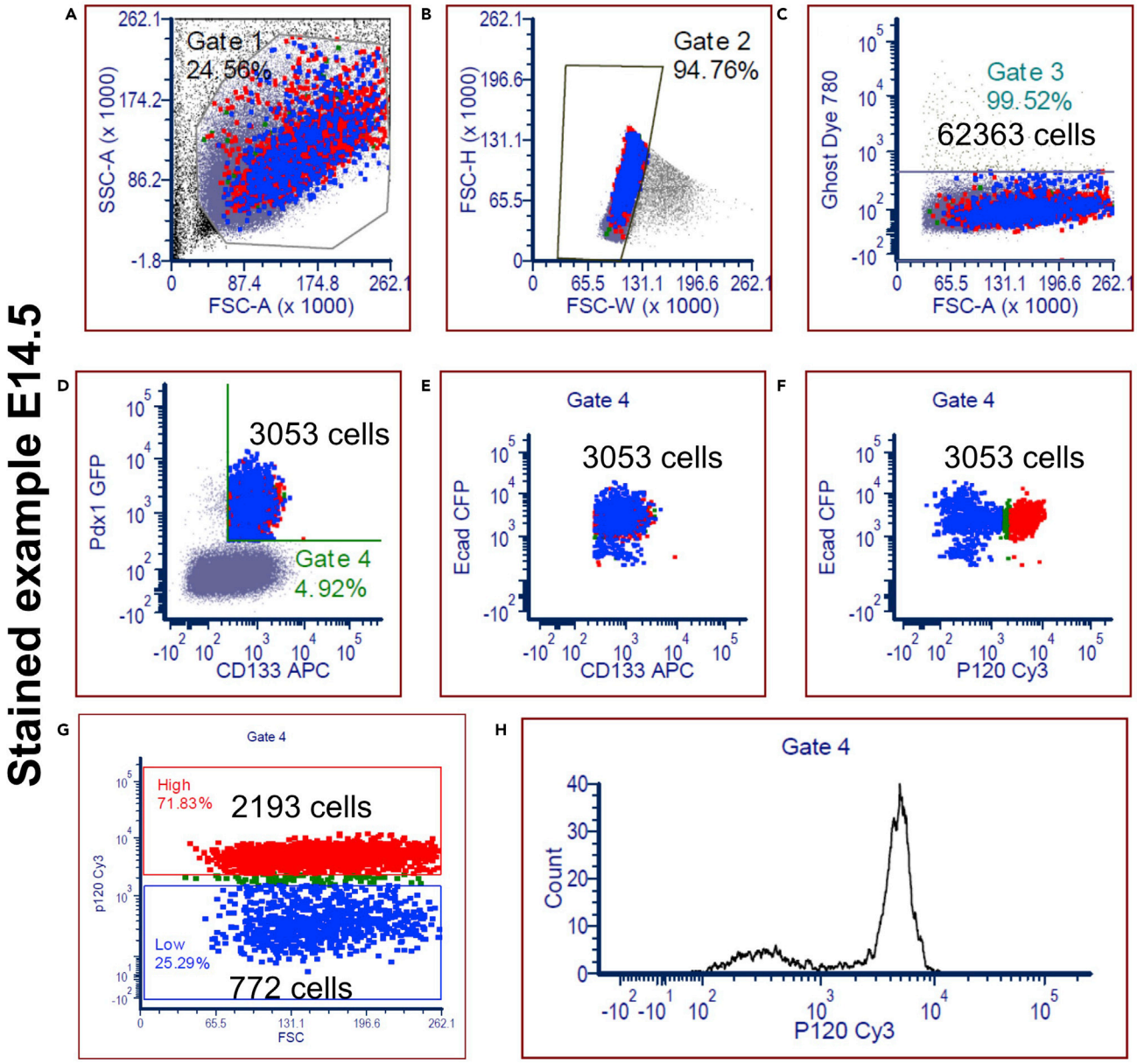

Figure 5. Representative data example of sample from a single E14.5 pancreas

The gating strategy, cell sorting and intensity diagram for P120CTN is included. Gate 1 (A) identifies the cells, gate 2 (B) excludes doublets and clumbs, gate 3 (C) excludes dead cells and gate 4 (D–G) identifies the pancreatic progenitor cells (3053 cells). A clearly distinguishable GFP+, APC+population is seen in D. Sorting in P120CTN high and low cells is shown in G, while the P120CTN expression profile is shown in H. Figure reprinted with permission from (Nyeng et al., 2019).

pancreatic progenitor marker CD133 and for P120CTN. Pancreatic organs were analyzed and sorted individually as shown in Figure 2. First, all events were gated based on light scattering properties to obtain only singlet cells (gate 1 and 2). Then these cells were gated based on Ghost dye staining to isolate the cells that were alive before fixation. Lastly, cells were gated for high Pdx1-GFP and CD133-APC expression to obtain the pancreatic progenitor cells and exclude CD133-APC negative, Pdx1-GFP positive endocrine cells. Analysis for P120CTN content revealed two clear populations, which were subsequently sorted (Figures 4 and 5).

LIMITATIONS

This protocol was optimized for use on embryonic pancreatic tissue. If used on adult pancreatic tissue, enzymatic auto-digestion may impede the isolation of intact cells. If used on other embryonic tissues, the digestion step 11–12 may need to be optimized further.

The maximum number of processed samples per experiment is dependent on how many embryos of a given genotype is obtained per dissected mouse. If very elaborate combinations of reporter strains are needed, the likelihood of obtaining samples with the correct genotype will be low. The maximum number of processed mice per experiment will depend on the experience of the researcher and the number of embryos per pregnant mouse. In our experience, we can process 3 pregnant mice per experiment.

This protocol has not been tested for detection of intracellular proteins with low expression (such as certain transcription factors). Note also that expression of some extracellular antigens will be impeded by the cell dissociation method (trypsin). An example of this is E-CADHERIN: E-CADHERIN was detected using a reporter mouse in this experiment, as antibody detection of the extracellular portion of E-CADHERIN would most likely be unsuccesfull due to the dissociation method (Blauvelt et al., 1995).

TROUBLESHOOTING

Below we list a number of potential problems. Some problems have several causes, which are described in numbered list format. The solutions suggested are numbered in a corresponding fashion, such that solution number 1 adresses cause number 1 and so forth.

Problem 1: Incomplete digestion

Incomplete digestion of tissue in step 11–12 will result in visible clumps of cells or tissue and will greatly affect downstream processing (see Problem 2)

Potential solution

Gentle trituration is critical for the digestion step, and should be performed exactly as described. Adjust the incubation times, if tissue is incompletely digested. No tissue should be visible after the digestion step. Modify the incubation time if a different tissue (non-pancreatic or adult) is used.

Problem 2: Low cell yields

Low cell yields (see Figure 6) can have several causes. The most common are:

1. Incomplete digestion of tissue leading to loss of cells in the filtration step (step 14)
2. Loss of cells during the washing steps (steps 15–19)
3. High cell death (see problem 3)

Potential solution

1. Gentle trituration is critical for the digestion step (steps 11 and 12), and should be performed exactly as described. Adjust the incubation times, if tissue is incompletely digested. No tissue should be visible after the digestion step. See also problem 1.
2. The protocol includes many washing steps (spin, aspirate supernatant and resuspend cells) and it is critical that the pellet is not disturbed. Some cell loss is inevitable: The pellet is visible during the early washing steps, but can be difficult to see during later washing steps. After spinning, the liquid should be removed very gently by pipetting, leaving a bit of liquid in the tube.
3. See problem 3

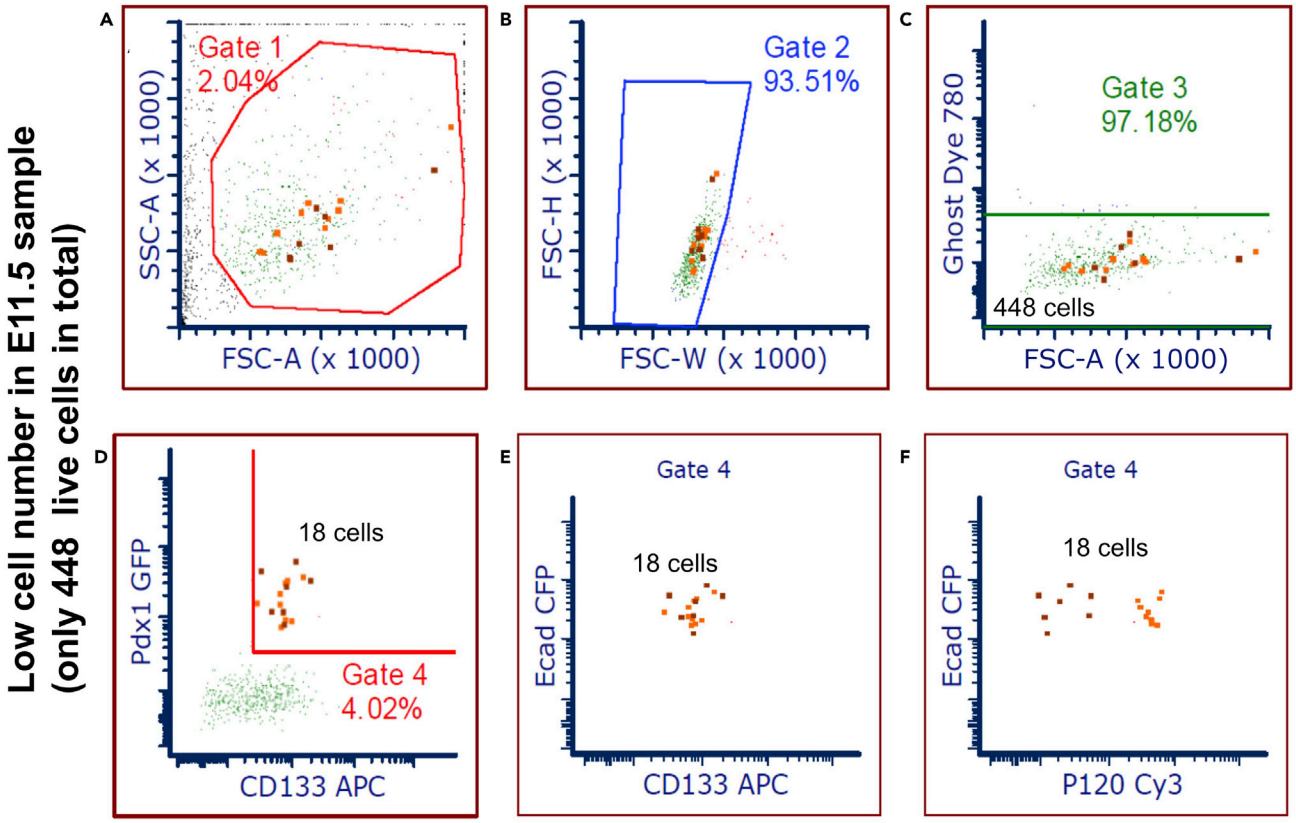

Figure 6. Data example of sample from a single E11.5 pancreas with extensive cell loss during processing

The gating strategy is shown. Gate 1 (A) identifies the cells, gate 2 (B) excludes doublets and clumbs, gate 3 (C) excludes dead cells and gate 4 (D–F) identifies the pancreatic progenitor cells (18 cells).

Problem 3: High cell death

A high amount of dead cells in the sample could be due to incorrect handling of cells prior to the fixation step (steps 6–16). We routinely detected less than 1% dead cells in our samples of embryonic pancreas (see Figures 3, 4, and 5), but other tissues could be more sensitive to handling.

Potential solutions

- Keep embryos, tissue and cells cold by using ice-cold solutions and keeping on wet ice as directed during steps 6–16, with exception of the digestion steps 11 and 12.
- Dissect tissue at a relatively fast pace (maximum 1 h dissection time per litter), ensuring embryos do not sit long between euthanizing the pregnant mouse and colleting the sample in ice-cold medium (steps 8 and 10). If possible work 2 persons on this part of the protocol to ensure a steady pace.
- Triturate very gently working at a slow, steady pace and never use a small size pipette tip (only P1000) during the digestion steps 11 and 12.
- Do not take breaks during steps 6–16 (before fixation).
- Do not centrifuge cells at a high rotor speed or vortex.
- If a high amount of dead cells persists, test if your cells are sensitive to the absence of FBS in step 15 and adjust as mentioned in note.

Problem 4: Low fluorescence

Low fluorescence can have several causes. The most common are:

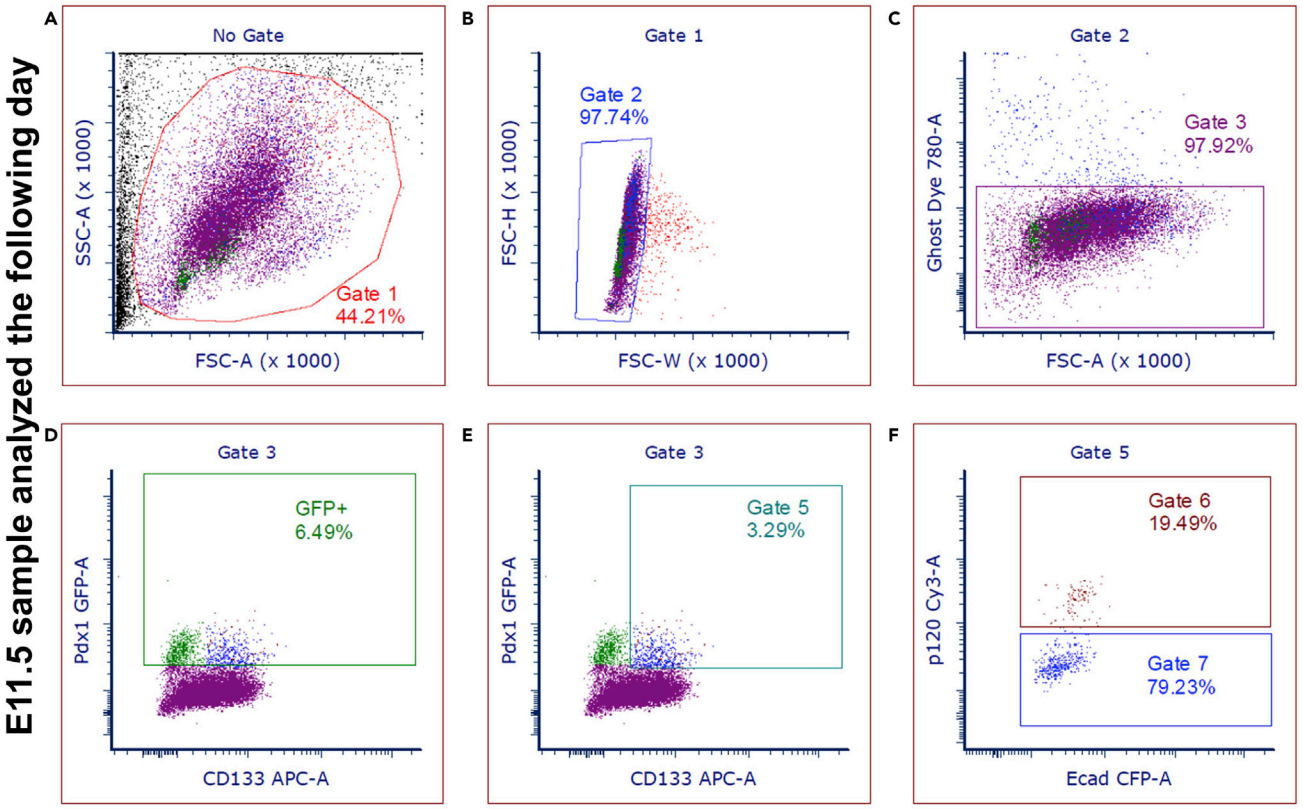

Figure 7. Data example of sample from a single E11.5 pancreas analyzed one day after processing

The gating strategy is shown. Gate 1 (A) identifies the cells, gate 2 (B) excludes doublets and clumbs, gate 3 (C) excludes dead cells and gate 4 (D–F) identifies the pancreatic progenitor cells. Loss of fluorescence is seen for GFP (PDX1), APC (CD133) and Cy3 (P120CTN), and there is no clearly distinguishable GFP+, APC+ population in D (compare to Figure 4).

1. Some cell surface antigens can be cleaved off by the enzymatic digestion with trypsin in step 12
2. Antibodies have low affinity for antigen
3. Improperly calibrated cytometer in step 21
4. The sample has lost fluorescence after staining (see Figure 7)

Potential solution

1. The antigens stained for in the exemplary protocol are compatible with trypsin digestion, but other antigens (such as cadherins and other proteins with cell surface epitopes) can be cleaved off by trypsin. In that case, the trypsin step could be omitted or replaced with a TrypLE digestion step (cat# 12605036, Thermo Fisher Scientific)
2. Antibodies should always be verified. Using an antibody that has been verified in another publication, by the vendor, or preferably in immunofluorescent staining of tissue sections for the particular tissue you are aiming to analyze, is recommended. If using other antibodies than those described in this protocol, different dilutions of the antibody should be tested as described in the section "Before you begin".
3. Ensure that optimal cytometer filters and settings are used, and consult with a cytometry specialist if further troubleshooting is needed
4. It is important that samples are analyzed immediately following staining (step 20c), or they may lose fluorescence intensity. Figure 7 shows cells analyzed the following day, resulting in lower fluorescent intensity for endogenous fluorescent proteins (GFP), and antibody conjugated fluorophores (APC and Cy3) (compare to Figure 4).

Problem 5: High noise/background

High noise and background can be due to:

1. Autofluorescence
2. Unspecific antibody binding
3. Spectral overlap between fluorophores
4. Dead cell/debris has not been gated out

Potential solution

1. Use the "no fluorophore" control to detect if there are any autofluorescent cells in your tissue, and to identify detectors with high autofluorescence. Avoid using those detectors for markers in the panel. In our hands, the formaldehyde fixed embryonic pancreas contained negligble autofluorescence in the chosen emission range (see Figure 3). Autofluorescence could be higher in other tissues and/or if using other fixatives. Autofluorescence will for example increase if the fixative is changed to glutaraldehyde (Lee et al., 2013)
2. Titrate antibody concentrations (see "Before you begin" step 1) and use internal negative control cells (cells in you sample which are known to not express your antigen), or isotype controls if available. Isotype controls should be carefully selected to not only be of the same isotype (heavy chain and light chain), but also to have the same fluorophore, the same ratio of fluorescent molecules/antibody, and to be produced in the same manner (buffers, purification etc) (Hulspas et al., 2009; Maecker and Trotter, 2006). Incomplete blocking could also cause unspecific antibody binding. 10% FBS is included in this protocol to block unspecific protein binding during staining. There is no Fc receptor blocking step, because we analyse cells from embryonic organs (pancreas), which do not to our knowledge contain Fc receptors. For users who wish to test our protocol on cell types which do express Fc receptors (mainly immune system cells), such a step should be included (Hulspas et al., 2009)
3. Use single color controls to compensate for spectral overlap (see step 22). See (Maecker and Trotter, 2006) for alternative strategies if appropriate single color controls are not available.
4. Use a fixable live/dead marker such as the Ghost Dyes to exclude dead cells from the analysis (see step 1))

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Pia Nyeng (pnyeng@ruc.dk).

Materials availability

All the materials used in this protocol are commercially available.

Data and code availability

Data are available upon request from the lead contact

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AUTHOR CONTRIBUTIONS

P.N. developed the methods and produced the example data for this protocol. G.V.D.C. contributed to method development and analysis of example data. P.N. wrote the first draft of the manuscript. G.V.D.C. edited the manuscript. H.S. acquired funding and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests

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