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# Flow cytometric analysis of the murine placenta to evaluate nanoparticle platforms during pregnancy

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ABSTRACT

Clinically approved therapeutics for obstetric conditions are extremely limited, with over 80% of drugs lacking appropriate labeling information for pregnant individuals. The pathology for many of these obstetric conditions can be linked to the placenta, necessitating the development of therapeutic platforms for selective drug delivery to the placenta. When evaluating therapeutics for placental delivery, literature has focused on *ex vivo* delivery to human placental cells and tissue, which can be difficult to source for non-clinical researchers. Evaluating *in vivo* drug delivery to the placenta using small animal models can be more accessible than using human tissue, but robust, quantitative methods to characterize delivery remain poorly established. Here, we report a flow cytometric method to evaluate *in vivo* drug delivery to the murine placenta. Specifically, we describe techniques to identify key cell types in the murine placenta — trophoblasts, endothelial cells, and immune cells — *via* flow cytometric analysis. While we have employed this method to detect lipid nanoparticle-mediated nucleic acid delivery, this approach can extend to a variety of drug carriers (*e.g.,* liposomes, exosomes, polymeric and metallic nanoparticles) and payloads (*e.g.,* small molecules, proteins, other nucleic acids). Similarly, we describe the application of this method toward immunophenotypic analysis to assess changes in the placental immune environment during disease or in response to a therapeutic. Together, the techniques reported herein aim to broaden the accessibility of placental research in an effort to encourage collaboration between physician-scientists, engineers, placental biologists, and clinicians for developing novel therapeutics to treat placental conditions during pregnancy.

## 1. Introduction

Women's health is a rapidly growing field in the science and medicine community as researchers, clinicians, and government agencies aim to bridge the disparity between disease burden and preclinical funding for women-specific disorders [1–3]. Of particular importance in the women's health field are obstetric conditions, as very few therapeutics have been rationally engineered and clinically approved to treat conditions during pregnancy. The placenta represents a unique organ target for obstetric conditions, as dysregulation in placental development and function is thought to be associated with a variety of obstetric conditions, including pre-eclampsia, fetal growth restriction, preterm birth, and the spectrum of placenta accreta disorders [4].

Nanotechnology-assisted drug delivery offers a unique therapeutic strategy for treating these obstetric conditions, as it helps overcome many of the barriers that exist to conventional delivery methods [1, 4–7]. Many studies of late have engineered novel drug delivery systems that enable local targeting to the placenta, including liposomes [8,9], lipid nanoparticles (LNPs) [10–14], and polymeric nanoparticles [15, 16]. Selective drug delivery to the placenta using platforms such as these could mean lower required drug doses to achieve therapeutic efficacy; this is a major design consideration for therapeutics during pregnancy,

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as adverse effects on the childbearer and developing fetus must be avoided [1].

To evaluate the efficacy of such novel therapeutics, a large body of literature exists on *in vitro* and *ex vivo* models for assessing placental delivery [17,18]. These include the traditional two-dimensional culture of immortalized trophoblast cell lines, transwell culture models with trophoblasts and/or endothelial cells for assessing drug transport across the placenta, and human placenta-derived trophoblasts, explants, and perfusion culture systems [17,19]. These models, particularly those utilizing three-dimensional systems derived from primary human placental samples, provide significant value for clinical translation given the differences in placental development and structure across species [1]. For example, human placentas take about 20 weeks to fully develop and are hemomonochorial in structure, with a single layer of syncytiotrophoblast at the maternal-fetal interface. However, rodent (*i.e.,* mouse, rat, and hamster) placentas are fully formed after 1–2 weeks and have a hemotrichorial structure in that two cytotrophoblast layers surround the syncytiotrophoblast layer. On the other hand, these *in vitro/ex vivo* models using human placental tissue also have limitations compared to preclinical *in vivo* models, particularly when considering the often-poor correlation between *in vitro* and preclinical *in vivo* performance of drug delivery systems [20]. Additionally, human placental tissue can be difficult to source for non-clinical researchers, who often rely on small animal models to evaluate the efficacy of nanoparticle therapeutics.

Preclinical *in vivo* models are particularly critical when assessing the placental tropism of therapeutics and their capacity to limit accumulation in the liver, spleen, or kidneys upon systemic administration [1]. Furthermore, evaluating nanoparticle (NP) delivery on a cellular level in the placenta — whether to trophoblasts, endothelial cells, or immune cells — is an important consideration, as cellular targets will likely vary based on the disorder and payload [4]. While flow cytometric analysis of human placenta-derived trophoblasts or tissue is well reported in literature [21,22], differences in cellular markers and procedure scalability for a large number of samples impede their direct application for flow cytometric analysis of the placenta in small animal models [23,24]. Additionally, as the field of placenta-tropic drug delivery systems is still in its infancy, methodologies to support accessibility and consistency across diverse teams of scientists is essential.

To address these challenges, we describe a method for flow cytometric analysis of the murine placenta for the evaluation of nanoparticle therapeutics for pregnancy disorders (Fig. 1). Specifically, the method outlines the procedure for murine placental tissue digestion, red blood cell lysis, and immunostaining of cell surface and intracellular markers to identify key cell types of interest, including endothelial cells, immune cells, and trophoblasts. Where possible, we note ways to increase the scalability of the procedure, an important consideration given that several placentas (*i.e.*, 5–12) can be isolated from a single mouse — the method reported here enables flow cytometric analysis of >150 mouse placentas in only 1–2 working days. Finally, we comment on the application and limitations of the method and compare the procedure to alternative methods for assessing local delivery of NPs to the placenta. The techniques reported herein aim to broaden the accessibility of placental research in an effort to encourage collaboration between physician-scientists, engineers, and clinicians for developing therapeutics to treat placental disorders during pregnancy.

## 2. Protocol

1. Prepare or purchase NPs as desired. For example, the placenta-tropic LNP formulation A4 can be formulated to encapsulate mCherry mRNA as previously described [10]. Briefly, the A4 ionizable lipid (Cayman Chemical cat. #38351) can be combined with the lipid excipients 1,2-dioleoyl-*sn*-glycero-2-phosphoethanolamine (DOPE, Avanti Polar Lipids cat. #850725), cholesterol (Sigma-Aldrich cat. #C8667), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (C14-$PEG_{2000}$, Avanti Polar Lipids cat. #880150) at relative molar ratios 35:16:46.5:2.5 to formulate LNPs *via* pipette or microfluidic mixing with an aqueous phase containing mCherry mRNA.
2. Administer NPs to time-dated pregnant female mice (*e.g.*, C57BL/6, ICR, BALB/c, CD-1) on the desired gestational day *via* intraperitoneal (*i.p.*) or intravenous (*i.v.*) injection with a maximum injection volume of 200 μL for both administration routes.

**Note 1**. The appropriate gestational day will vary depending on the intended application of the experiment, with literature guiding the comparison of the developmental timeline of mouse pregnancy to human pregnancy [25]. Clean dissection of the placenta is possible beginning around gestational day E11 with the development of the mature placenta [26].

**Note 2**. NP dosages are typically calculated on a weight of carrier/cargo per weight of animal basis. The weight of time-dated pregnant mice varies substantially across gestational day and litter size.

**Note 3**. The appropriate administration route will also vary depending on the intended application of the experiment, with some previous literature suggesting that NP delivery to the placenta is feasible under both *i.p.* and *i.v.* administration routes [13]. It is important to consider the optimal nanoparticle administration route for the intended clinical application, which will likely depend on numerous factors, including NP retention and clearance and degree of off-target delivery.

3. At the desired time post-administration, euthanize mice in a chamber with a $CO_2$ fill rate of 30–70% of the chamber volume per minute. Dissect the mouse, removing the intact uterine horn from the abdominal cavity and placing it into a 90 mm petri dish filled with 1╳ PBS. Individually dissect the placentas from the uterine horn, rinsing each placenta before individually placing them in 5 mL tubes pre-filled with 2 mL of 1╳ DNase I Reaction Buffer (diluted 10╳ in deionized water, New England BioLabs cat. #B0303S). Keep tubes on ice until further processing.

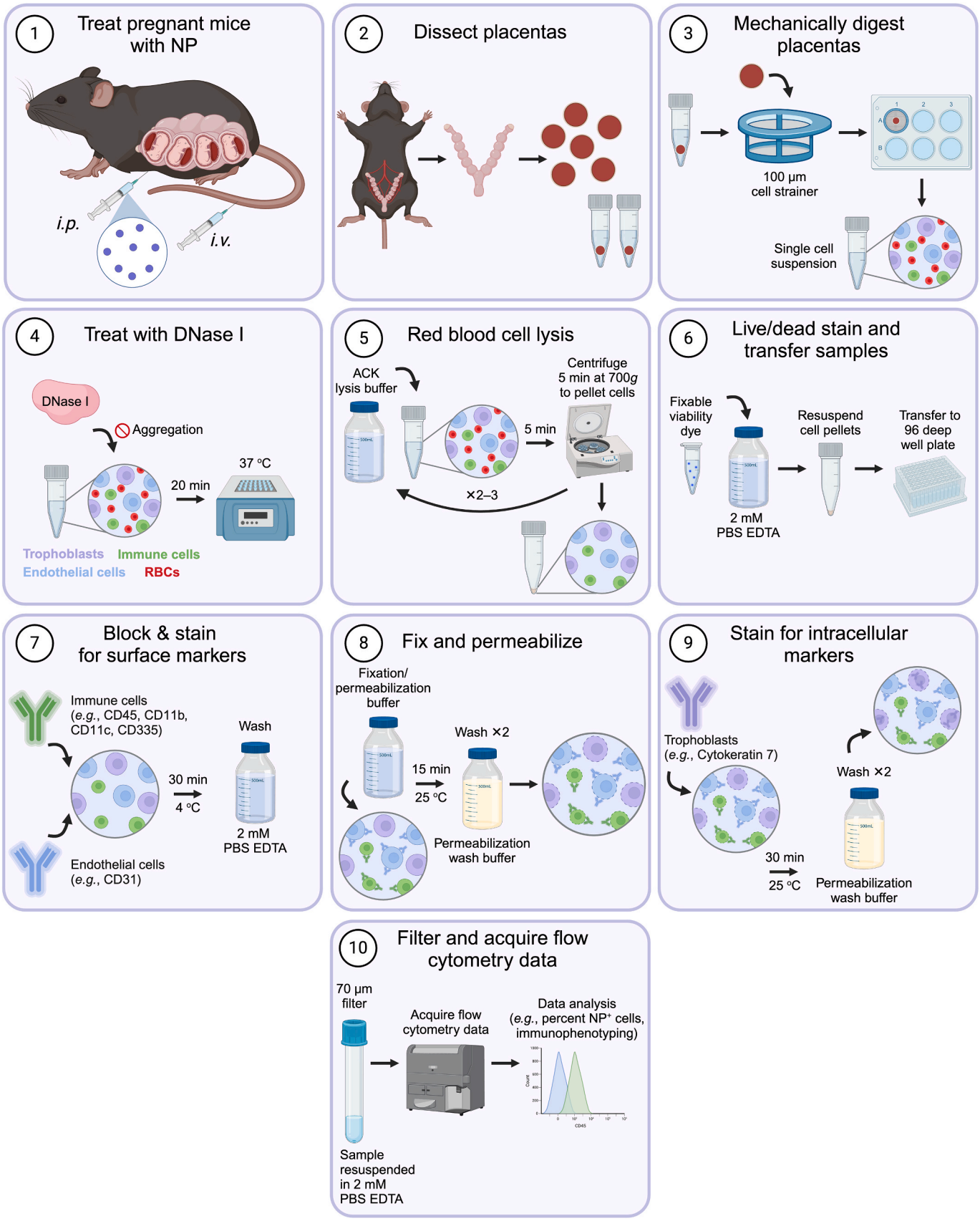
**Note 4**. To increase statistical power, we recommend processing each placenta individually, enabling data collection from a series of technical replicates (*i.e.*, each placenta) from each biological replicate or mouse. Otherwise, 2–3 placentas can be pooled for subsequent processing.

4. Mechanically digest each placenta using a cell strainer (100 μm pore mesh size, Fisher Scientific cat. #22-363-549) placed into a well of a 6-well plate for each placenta. Invert the tube containing each placenta in 2 mL of 1× DNase I Reaction Buffer onto the cell strainer mesh and push the tissue through using the rubber stopper end of a plunger removed from a 3 mL plastic syringe. Thoroughly rinse the cell strainer using the flow-through collected in the 6-well plate. Remove the cell suspension and return it to the 5 mL tube. Keep samples on ice until all placentas have been mechanically digested.

**Note 5**. Here, we recommend mechanical digestion using a cell strainer with a 100 μm pore mesh size to ensure sufficient yields of all cell types, including endothelial cells, which can be up to 50 μm in length. Care should be taken (*i.e.*, mixing samples manually with a pipette) to ensure cells are well suspended in solution to limit the formation of small cell clusters.

5. To limit cellular aggregation caused by exposed genomic DNA, add 20 μL of DNase I (New England BioLabs cat. #M0303L) to a final concentration of 20 units/mL to each tube of placental cell suspensions. Let digestion proceed for 20 min at 37 °C with gentle shaking at 300 rpm.

# Flow cytometric analysis of the murine placenta




*(caption on next page)*

**Fig. 1.** Procedure for flow cytometric analysis of the murine placenta to identify drug delivery platforms for treating pregnancy disorders. Pregnant mice are treated with nanoparticles (NPs), after which placentas are dissected from the mouse and digested to generate single cell suspensions. Cells are treated with DNase I to limit cellular aggregation and lysis buffer to remove red blood cells (RBCs). To identify key cell populations of interest, samples are stained with a fixable viability dye, blocked, and stained for cell surface markers, including those to identify immune and endothelial cells. Then, cells can be fixed and permeabilized to analyze intracellular targets, including cytokeratin 7 as a marker for trophoblasts. Finally, data are acquired on a flow cytometer and analysis is performed to identify the fraction of NP-positive cells, changes in immune infiltration, *etc*. ACK: ammonium-chloride-potassium; EDTA: ethylenediaminetetraacetic acid. Made with BioRender.com.

**Note 6.** If substantial cellular aggregation is observed, further enzymatic digestion with collagenase type I [27,28] can be exploited to remove collagens found in the placental matrix that might promote cell-cell adhesion. Optimal digestion times should balance the need for sufficient enzymatic digestion with preserving cellular viability.

6. To remove red blood cells, add 2 mL of ammonium-chloride-potassium (ACK) lysing buffer (Gibco cat. #A1049201) to each tube and incubate for 5 min at room temperature. Centrifuge tubes at 700*g* for 5 min and remove supernatant using a vacuum aspirator. Repeat 1–2 additional times as needed until supernatant is clear and the cell pellet is no longer red.
7. Prepare a 1:100 dilution of a fixable viability dye (*e.g.*, Zombie UV, BioLegend cat. #423107) in 1╳ PBS with 2 mM ethylenediaminetetraacetic acid (EDTA). Resuspend the cell pellets in 0.5 mL of diluted viability dye and transfer to round bottom, 96 deep-well plates. Incubate cells at room temperature, in the dark, for 15 min.

**Note 7.** 1╳ PBS with 2 mM EDTA is used throughout, where possible, in an effort to chelate metal ions and limit cell-cell adhesion mediated by cell adhesion molecules (CAMs).

**Note 8.** Transferring samples to a 96 deep-well plate is intended to increase throughput of the remaining staining and washing steps.

8. Wash cells by adding 1 mL of 1╳ PBS with 2 mM EDTA to each sample/well; this can be performed using a multi-channel or repeat pipettor. Centrifuge the 96 deep-well plate(s) at 700*g* for 5 min and remove the supernatant using a vacuum aspirator. Ensure the centrifuge is well balanced with a second 96 deep-well plate.
9. Prepare a 1:200 dilution of TruStain FcX PLUS (anti-mouse CD16/32 antibody, BioLegend cat. #156604) in 1╳ PBS with 2 mM ethylenediaminetetraacetic acid (EDTA) to create blocking buffer. Resuspend the cell pellets in 0.5 mL of blocking buffer and let cells block for 5–10 min.
10. Stain cell surface markers using fluorophore-conjugated anti-mouse CD45 and anti-mouse CD31 antibodies to identify immune cells and endothelial cells, respectively. Other cell surface markers to identify additional immune cell subpopulations (*e.g.*, CD19, CD3, CD11b, CD11c, CD335) can also be used depending on the application of the experiment. For all cell surface markers, create a "master mix" of antibodies using approximately 1–2 μg of each antibody per sample; stain cells with the master mix for 30 min in the dark at 4 °C.

**Note 9.** After staining cell surface markers, samples will be fixed and permeabilized to enable intracellular staining. With this in mind, it is important to consider the fixation stability of the selected fluorophores for staining cell surface markers. For example, protein-based dyes such as PE and APC, and especially their tandem dyes (PE-Cyanine 7 and APC-Cyanine 7), are susceptible to quenching of fluorescence signal upon fixation [29]. Synthetic dyes such as Alexa Fluor and Brilliant Violet series fluorophores are more robustly resilient to fixation.

**Note 10.** By creating a "master mix" of antibodies, each sample need only be stained once, increasing experimental throughput. For example, we have previously used Brilliant Violet 421 anti-mouse CD45 antibody at a concentration of 0.2 μg/μL (BioLegend cat. #103134) and FITC anti-mouse CD31 antibody at a concentration of 0.5 μg/μL (BioLegend cat. #102506). For 1 μg of each antibody, a master mix would require 5 μL of the CD45 antibody and 2 μL of the CD31 antibody per sample; the samples would then each be stained with 7 μL of the master mix.

**Note 11.** To avoid mixing each well individually after adding the master mix, the 96 deep-well plate(s) can be placed on a microplate shaker for 5 min at 300 rpm.

**Note 12.** This procedure is written assuming fluorophore-conjugated primary antibodies are being used in order to minimize rinse steps. If unconjugated primary antibodies are to be used with fluorophore-conjugated secondary antibodies, stain cells with the primary antibody as described above, thoroughly wash cells following the instructions provided in step 8, and stain cells with the secondary antibody.

11 Prepare and stain compensation controls; the total number of compensation controls required is equal to the number of fluorophores in the panel plus one additional unstained control (to aid in determining background autofluorescence) (Table 1). A fraction (about one-half) of the cell suspension generated from one mouse placenta can be used for these controls. For most compensation controls, cell suspensions from untreated mice should be stained individually with each fluorophore in the panel. Generating compensation controls for the NP fluorophore (s) can be more complicated. In the case of a single NP-associated fluorophore, a cell suspension from a NP-treated mouse can serve as an appropriate compensation control. If the NP is associated with more than one fluorophore (*e.g.*, both the cargo and NP are fluorescently labeled), generating compensation controls would require treating a mouse with separately prepared NPs containing only one of the fluorophores, repeating this for the total number of NP-associated fluorophores.

**Note 13.** Alternatively, compensation controls for NP fluorophore(s) can be generated by individually staining/treating immortalized cells or by using UltraComp eBeads Compensation Beads (Invitrogen cat. #01-2222-41) that can be stained with fluorophore-conjugated antibodies. Additionally, calibration beads are also available if using reporter mRNA cargoes encoding the mCherry or GFP fluorescent proteins (Takara cat. #632595 and #632594). When acquiring compensation and experimental data on the flow cytometer, it is important to keep in mind that the light scattering characteristics of immortalized cells (particularly unfixed) or compensation beads will likely differ from those of murine placental cell suspensions.

12. Similarly, we recommend preparing fluorescence-minus one (FMO) controls; the total number of FMOs required is equal to the number of fluorophores in the panel (Table 1). For FMOs, cell suspensions from either untreated or treated mice should be stained with every fluorophore except one, and this should be repeated for all fluorophores in the panel. Again, if the NP is associated with more than one fluorophore, one mouse would need to be treated with separately prepared NPs containing only one fluorophore, repeating this process for the total number of NP-associated fluorophores.

**Table 1**
Compensation and fluorescence-minus one (FMO) controls for an example flow cytometry panel [10] used to assess lipid nanoparticle (LNP)-mediated delivery of a reporter mRNA cargo encoding the mCherry red fluorescent protein to the murine placenta.

| Compensation controls | | |
|---|---|---|
| Comp. control | Experimental sample | Fluorophore/Dye |
| Zombie UV-Live/Dead | Untreated | Zombie UV |
| Brilliant Violet 421-CD45 | Untreated | Brilliant Violet 421 |
| FITC-CD31 | Untreated | FITC |
| Alexa Fluor 700-Cytokeratin 7 | Untreated | Alexa Fluor 700 |
| mCherry-LNP | LNP treated | mCherry |
| Unstained | Untreated | None |

| Fluorescence-minus one (FMO) controls | | |
|---|---|---|
| FMO | Experimental sample | Fluorophores/Dyes |
| Zombie UV-Live/Dead | LNP treated | Brilliant Violet 421, FITC, Alexa Fluor 700 |
| Brilliant Violet 421-CD45 | LNP treated | Zombie UV, FITC, Alexa Fluor 700 |
| FITC-CD31 | LNP treated | Zombie UV, Brilliant Violet 421, Alexa Fluor 700 |
| Alexa Fluor 700-Cytokeratin 7 | LNP treated | Zombie UV, Brilliant Violet 421, FITC |
| mCherry-LNP | Untreated | Zombie UV, Brilliant Violet 421, FITC, Alexa Fluor 700 |

**Note 14**. Similarly to compensation controls, FMOs can alternatively be generated by staining immortalized cells or Compensation Beads with all but one of the fluorophore-conjugated antibodies in the panel.

13. Repeat step 8 to wash cells.
14. Resuspend the cell pellets in 100 μL of 1╳ PBS with 2 mM EDTA. Make sure the cells are well suspended by thoroughly mixing each well — this can be completed quickly by using a multichannel pipette. Add 150 μL of Cyto-Fast Fix/Perm Solution (BioLegend cat. #426803) to each well, mix thoroughly, and incubate for 15 min at room temperature on a microplate shaker at 300 rpm.

**Note 15**. Ensure cells are thoroughly mixed; if cells are not well suspended, they will become permanently aggregated upon fixation.

**Note 16**. It is important to fix and permeabilize all experimental samples in addition to all compensation controls and FMOs, as these processes can alter light scattering characteristics during flow cytometry.

15. Add 1 mL of 1╳ Cyto-Fast Perm Wash Solution (BioLegend cat. #426803, diluted 10╳ in deionized water), centrifuge the 96 deep-well plate(s) at 700*g* for 5 min and remove the supernatant using a vacuum aspirator.
16. Resuspend the cell pellets in 100 μL of 1╳ Cyto-Fast Perm Wash Solution. Stain intracellular markers using fluorophore-conjugated anti-mouse cytokeratin 7 antibody to identify trophoblasts. As with cell surface markers, use 1–2 μg of antibody for each experimental sample. Stain compensation controls and FMOs. Let all samples stain for 30 min in the dark at room temperature.

**Note 17**. While staining for intracellular markers, it is critical that cells are suspended in 1╳ Cyto-Fast Perm Wash Solution to ensure sufficient antibody permeation into cells for binding intracellular targets.

**Note 18**. Again, to avoid mixing each well individually after adding antibody, the 96 deep-well plate(s) can be placed on a microplate shaker for 5 min at 300 rpm.

17. Repeat step 8 to wash cells.
18. Resuspend samples in 0.5 mL of 1╳ PBS with 2 mM EDTA and transfer to 5 mL flow tubes fitted with filtered (70 μm pore mesh size) caps. Acquire data on a flow cytometer, adjusting gains to ensure immune cells, endothelial cells, and trophoblasts are on scale, as they often vary in size and complexity.

**Note 19**. As cells have already been fixed to enable intracellular staining, data can be acquired the next day if necessary.

19. Analyze data using FMO controls to set thresholds for positivity including, for example, the percent NP-positive cells or the proportion of a given immune cell subtype out of $CD45^+$ cells.

## 3. Applications and limitations of the method

Upon systemic administration, NPs will tend to accumulate primarily in the liver due to a first-pass hepatic clearance effect [30]. In an effort to engineer nanoparticle therapeutics for treating placental disorders, previous work by our group and others has explored several techniques for enhancing local nanoparticle delivery to the placenta. These include altering the chemical structures of nanoparticle excipients [10–12] and functionalizing the nanoparticle surface with various targeting moieties, such as peptides and antibodies, to promote uptake by placental cells [9,14]. To date, the biodistribution of these nanoparticle platforms has been evaluated primarily in small animal models such as pregnant mice. The method described here can be employed for quantifying such NP delivery to the key cell types in the murine placenta through fluorescent labeling of the NP itself and/or encapsulation of a fluorescent molecule or fluorescent protein-encoding nucleic acid cargo. Additionally, the procedure can be applied to different NP platforms (*e.g.*, lipid, polymeric, and metallic NPs, drug conjugates) and payloads (*e.g.*, small molecules, proteins, nucleic acids), enabling quantification of the fraction of NP- or cargo-positive cells.

To further translate NP therapies for placental disorders to the clinic, it will be essential to assess their capacity for safe, local delivery to the placenta in larger animal models such as non-human primates, whose gestational periods and placental structures more closely model those of humans. It is also important to consider the cardiovascular changes that occur during pregnancy and their potential role on NP distribution and clearance. For example, by 24 weeks of gestation in human pregnancy, cardiac output increases by 45%, with 20–25% of this output representing blood flow to the uterus and placenta [31,32]. While there is little literature evaluating NP pharmacokinetics in pregnant humans and non-human primates, these evaluations are essential for safely translating NP therapeutics to treat pregnant patients in the clinic.

Besides NP delivery, this method can be used to evaluate changes in the local placental microenvironment in disease models or in response to novel therapeutics. For example, as placental immune dysregulation is associated with many disorders during pregnancy, assessing immune cell infiltration and activation in the placenta would be essential to limit exacerbation of a preexisting disease phenotype [33–35]. Similarly, the procedure described here can be used to assess changes in cell phenotype as well as differentiation across gestation and in various disease states, such as those that model pre-eclampsia, fetal growth restriction, pre-term birth, and recurrent pregnancy loss.

Where possible, we have noted opportunities to increase the scalability of the method, an important consideration given the large number of placental samples that can be collected from each mouse. In the procedure, we recommend processing each placental sample individually, enabling data collection from several technical replicates (*i.e.*, placentas) per mouse and increasing statistical power for subsequent

data analysis. Alternatively, several placentas from each mouse can be pooled and processed in aggregate, further increasing the throughput of the method if numerous experimental groups are being evaluated simultaneously. Individually dissecting and processing many placentas is, admittedly, rather labor intensive and time-consuming, a limitation of the current procedure. Other limitations of the method include those associated with flow cytometry experiments, including the cost of antibodies, access to an expensive instrument with sufficient lasers and filters for the intended number of fluorophores, and the difficulty of sample preparation and acquiring high-quality flow cytometry data for cell suspensions generated from tissues.

## 4. Comparison to alternative methods

While previously published protocols have provided methods for isolating mouse placental hematopoietic stem cells [36], leukocytes [37], and endothelial cells [28,38], this protocol aims to enable isolation and flow cytometric analysis of several murine placental cell types simultaneously including trophoblasts, endothelial cells, and immune cells. Besides flow cytometric analysis, alternative methods can be used to assess NP delivery to the placenta. For example, studies have employed *in vivo* imaging systems (IVIS) where mice are euthanized and placentas are either left inside or dissected from the uterine horn for fluorescent or bioluminescent imaging [10–12,39,40]. This technique is useful for rapid discovery of NP therapeutics for placental delivery, requiring no sample processing following dissection from the mouse. However, delivery can only be quantified at the tissue level and detection of a small amount of fluorescent NP or cargo can be rather difficult due to constraints on fluorescence signal-to-noise ratio in biological samples [41].

Alternatively, many groups have used microscopy-based immunofluorescence approaches where placentas are dissected from a mouse, fixed, and processed for histology to detect local NP delivery to the placenta [9,39,40,42]. By analyzing fluorescence colocalization in image processing software, this method can be used to analyze cell-specific delivery of a NP therapeutic, including the ability to evaluate the effect of tissue microenvironment on NP delivery. While this approach is an improvement on IVIS-based techniques, quantifying NP delivery using a single tissue slice does not necessarily represent the whole placenta, and selecting representative images can be a subjective process. These immunofluorescence techniques are particularly useful for analyzing NP delivery in the placental labyrinth where trophoblast cells fuse to form a continuous, multi-nucleated syncytium. While quantifying NP delivery to these syncytiotrophoblast cells using flow cytometry might be challenging, alterations to the current method to limit cell rupture during mechanical dissociation could be employed. Imaging flow cytometry is another potentially useful technique for assessing NP delivery to placental syncytiotrophoblasts, as it has shown promise for single-cell analysis of multinucleated cells [43].

## 5. Conclusion

In summary, we report a method for flow cytometric analysis of the murine placenta, a technique that can be applied for developing new therapeutics for delivery to the placenta. Procedures such as the one reported here can broaden the accessibility of placental research and encourage collaboration within the science and medicine community. This will facilitate efforts to develop new therapeutic strategies for treating a range of obstetric disorders including pre-eclampsia, fetal growth restriction, preterm birth, and the spectrum of placenta accreta disorders.

## CRediT authorship contribution statement

**Kelsey L. Swingle:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Alex G. Hamilton:** Writing – review & editing, Methodology, Conceptualization. **Michael J. Mitchell:** Writing – review & editing, Supervision, Methodology, Conceptualization.

## Declaration of Competing interest

K.L.S. and M.J.M. have filed a patent application related to this work.

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## References

[1] K.L. Swingle, A.S. Ricciardi, W.H. Peranteau, M.J. Mitchell, Delivery technologies for women's health applications, Nat Rev Bioeng (2023) 1–18, https://doi.org/10.1038/s44222-023-00040-w.
[2] A.A. Mirin, Gender disparity in the funding of diseases by the U.S. National Institutes of health, Journal of Women's Health 30 (2021) 956–963, https://doi.org/10.1089/jwh.2020.8682.
[3] R. VanKeulen-Miller, O.S. Fenton, Messenger RNA therapy for female reproductive health, Mol. Pharmaceutics (2024), https://doi.org/10.1021/acs.molpharmaceut.3c00803.
[4] H.C. Geisler, H.C. Safford, M.J. Mitchell, Rational design of nanomedicine for placental disorders: birthing a new era in women's reproductive health, Small (2023) 2300852, https://doi.org/10.1002/smll.202300852.
[5] N. Chaudhary, A.N. Newby, K.A. Whitehead, Non-viral RNA delivery during pregnancy: opportunities and challenges, Small n/a (2023) 2306134, https://doi.org/10.1002/smll.202306134.
[6] M.J. Mitchell, M.M. Billingsley, R.M. Haley, M.E. Wechsler, N.A. Peppas, R. Langer, Engineering precision nanoparticles for drug delivery, Nat. Rev. Drug Discov. (2020) 1–24, https://doi.org/10.1038/s41573-020-0090-8.
[7] A.G. Hamilton, K.L. Swingle, M.J. Mitchell, Biotechnology: overcoming biological barriers to nucleic acid delivery using lipid nanoparticles, PLoS Biol. 21 (2023) e3002105, https://doi.org/10.1371/journal.pbio.3002105.
[8] A.A. Alfaifi, R.S. Heyder, E.R. Bielski, R.M. Almuqbil, M. Kavdia, P.M. Gerk, S.R. P. da Rocha, Megalin-targeting liposomes for placental drug delivery, J. Contr. Release 324 (2020) 366–378, https://doi.org/10.1016/j.jconrel.2020.05.033.
[9] A. King, C. Ndifon, S. Lui, K. Widdows, V.R. Kotamraju, L. Agemy, T. Teesalu, J. D. Glazier, F. Cellesi, N. Tirelli, J.D. Aplin, E. Ruoslahti, L.K. Harris, Tumor-homing peptides as tools for targeted delivery of payloads to the placenta, Sci. Adv. 2 (2021) e1600349, https://doi.org/10.1126/sciadv.1600349.
[10] K.L. Swingle, H.C. Safford, H.C. Geisler, A.G. Hamilton, A.S. Thatte, M. M. Billingsley, R.A. Joseph, K. Mrksich, M.S. Padilla, A.A. Ghalsasi, M.-G. Alameh, D. Weissman, M.J. Mitchell, Ionizable lipid nanoparticles for in vivo mRNA delivery to the placenta during pregnancy, J. Am. Chem. Soc. 145 (2023) 4691–4706, https://doi.org/10.1021/jacs.2c12893.
[11] H.C. Safford, K.L. Swingle, H.C. Geisler, A.G. Hamilton, A.S. Thatte, A.A. Ghalsasi, M.M. Billingsley, M.-G. Alameh, D. Weissman, M.J. Mitchell, Orthogonal design of experiments for engineering of lipid nanoparticles for mRNA delivery to the placenta, Small n/a (2023) 2303568, https://doi.org/10.1002/smll.202303568.
[12] R.E. Young, K.M. Nelson, S.I. Hofbauer, T. Vijayakumar, M.-G. Alameh, D. Weissman, C. Papachristou, J.P. Gleghorn, R.S. Riley, Systematic development of ionizable lipid nanoparticles for placental mRNA delivery using a design of experiments approach, Bioact. Mater. 34 (2024) 125–137, https://doi.org/10.1016/j.bioactmat.2023.11.014.
[13] N. Chaudhary, A.N. Newby, M.L. Arral, S.S. Yerneni, S.T. LoPresti, R. Doerfler, D. M.S. Petersen, C. Montoya, J.S. Kim, B. Fox, T. Coon, A. Malaney, Y. Sadovsky, K. A. Whitehead, Lipid nanoparticle structure and delivery route during pregnancy dictate mRNA potency, immunogenicity, and maternal and fetal outcomes, Proc. Natl. Acad. Sci. USA 121 (2024) e2307810121, https://doi.org/10.1073/pnas.2307810121.
[14] H.C. Geisler, A.A. Ghalsasi, H.C. Safford, K.L. Swingle, A.S. Thatte, A.J. Mukalel, N. Gong, A.G. Hamilton, E.L. Han, B.E. Nachod, M.S. Padilla, M.J. Mitchell, EGFR-targeted ionizable lipid nanoparticles enhance *in vivo* mRNA delivery to the placenta, J. Contr. Release 371 (2024) 455–469, https://doi.org/10.1016/j.jconrel.2024.05.036.
[15] L. Li, H. Li, J. Xue, P. Chen, Q. Zhou, C. Zhang, Nanoparticle-mediated simultaneous downregulation of placental Nrf2 and sFlt1 improves maternal and fetal outcomes in a preeclampsia mouse model, ACS Biomater. Sci. Eng. 6 (2020) 5866–5873, https://doi.org/10.1021/acsbiomaterials.0c00826.
[16] J. Yu, J. Jia, X. Guo, R. Chen, L. Feng, Modulating circulating sFlt1 in an animal model of preeclampsia using PAMAM nanoparticles for siRNA delivery, Placenta 58 (2017) 1–8, https://doi.org/10.1016/j.placenta.2017.07.360.

[17] C.G. Figueroa-Espada, S. Hofbauer, M.J. Mitchell, R.S. Riley, Exploiting the placenta for nanoparticle-mediated drug delivery during pregnancy, Adv. Drug Deliv. Rev. 160 (2020) 244–261, https://doi.org/10.1016/j.addr.2020.09.006.
[18] P. Calis, L. Vojtech, F. Hladik, M.G. Gravett, A review of ex vivo placental perfusion models: an underutilized but promising method to study maternal-fetal interactions, J. Matern. Fetal Neonatal Med. 35 (2022) 8823–8835, https://doi.org/10.1080/14767058.2021.2005565.
[19] K. Orendi, V. Kivity, M. Sammar, Y. Grimpel, R. Gonen, H. Meiri, E. Lubzens, B. Huppertz, Placental and trophoblastic in vitro models to study preventive and therapeutic agents for preeclampsia, Placenta 32 (2011) S49–S54, https://doi.org/10.1016/j.placenta.2010.11.023.
[20] K. Paunovska, C.D. Sago, C.M. Monaco, W.H. Hudson, M.G. Castro, T.G. Rudoltz, S. Kalathoor, D.A. Vanover, P.J. Santangelo, R. Ahmed, A.V. Bryksin, J.E. Dahlman, A direct comparison of in vitro and in vivo nucleic acid delivery mediated by hundreds of nanoparticles reveals a weak correlation, Nano Lett. 18 (2018) 2148–2157, https://doi.org/10.1021/acs.nanolett.8b00432.
[21] J. Maldonado-Estrada, E. Menu, P. Roques, F. Barré-Sinoussi, G. Chaouat, Evaluation of Cytokeratin 7 as an accurate intracellular marker with which to assess the purity of human placental villous trophoblast cells by flow cytometry, J. Immunol. Methods 286 (2004) 21–34, https://doi.org/10.1016/j.jim.2003.03.001.
[22] J. Che, Z. Zheng, M. Li, X. Yao, Macrophage polarization in placenta accreta and macrophage-trophoblast interactions, American Journal of Reproductive Immunology 88 (2022) e13611, https://doi.org/10.1111/aji.13611.
[23] O. Farah, C. Nguyen, C. Tekkatte, M.M. Parast, Trophoblast lineage-specific differentiation and associated alterations in preeclampsia and fetal growth restriction, Placenta 102 (2020) 4–9, https://doi.org/10.1016/j.placenta.2020.02.007.
[24] A.K. Wakeland, F. Soncin, M. Moretto-Zita, C.-W. Chang, M. Horii, D. Pizzo, K. K. Nelson, L.C. Laurent, M.M. Parast, Hypoxia directs human extravillous trophoblast differentiation in a hypoxia-inducible factor–dependent manner, Am. J. Pathol. 187 (2017) 767–780, https://doi.org/10.1016/j.ajpath.2016.11.018.
[25] C.A. Waker, M.R. Kaufman, T.L. Brown, Current state of preeclampsia mouse models: approaches, relevance, and standardization, Front. Physiol. 12 (2021) 681632, https://doi.org/10.3389/fphys.2021.681632.
[26] K.A. Pennington, J.M. Schlitt, L.C. Schulz, Isolation of primary mouse trophoblast cells and trophoblast invasion assay, JoVE (2012) e3202, https://doi.org/10.3791/3202.
[27] K. Ottersbach, E. Dzierzak, Analysis of the mouse placenta as a hematopoietic stem cell niche, in: C.W. Eric So (Ed.), Leukemia: Methods and Protocols, Humana Press, Totowa, NJ, 2009, pp. 335–346, https://doi.org/10.1007/978-1-59745-418-6_17.
[28] I. Sandovici, M. Reiterer, M. Constância, C.M. Branco, Protocol to isolate and culture primary mouse feto-placental endothelial cells, STAR Protocols 3 (2022) 101721, https://doi.org/10.1016/j.xpro.2022.101721.
[29] J.D. Lifson, D.T. Sasaki, E.G. Engelman, Utility of formaldehyde fixation for flow cytometry and inactivation of the AIDS associated retrovirus, J. Immunol. Methods 86 (1986) 143–149, https://doi.org/10.1016/0022-1759(86)90278-4.
[30] Y.-N. Zhang, W. Poon, A.J. Tavares, I.D. McGilvray, W.C.W. Chan, Nanoparticle–liver interactions: cellular uptake and hepatobiliary elimination, J. Contr. Release 240 (2016) 332–348, https://doi.org/10.1016/j.jconrel.2016.01.020.
[31] M.C. Frederiksen, Physiologic changes in pregnancy and their effect on drug disposition, Semin. Perinatol. 25 (2001) 120–123, https://doi.org/10.1053/sper.2001.24565.
[32] M. Sanghavi, J.D. Rutherford, Cardiovascular physiology of pregnancy, Circulation 130 (2014) 1003–1008, https://doi.org/10.1161/CIRCULATIONAHA.114.009029.
[33] I. Aneman, D. Pienaar, S. Suvakov, T.P. Simic, V.D. Garovic, L. McClements, Mechanisms of key innate immune cells in early- and late-onset preeclampsia, Front. Immunol. 11 (2020). https://www.frontiersin.org/articles/10.3389/fimmu.2020.01864. (Accessed 11 January 2023).
[34] M.M. Faas, P. De Vos, Innate immune cells in the placental bed in healthy pregnancy and preeclampsia, Placenta 69 (2018) 125–133, https://doi.org/10.1016/j.placenta.2018.04.012.
[35] I. Yougbaré, W.-S. Tai, D. Zdravic, B.E. Oswald, S. Lang, G. Zhu, H. Leong-Poi, D. Qu, L. Yu, C. Dunk, J. Zhang, J.G. Sled, S.J. Lye, J. Brkić, C. Peng, P. Höglund, B. A. Croy, S.L. Adamson, X.-Y. Wen, D.J. Stewart, J. Freedman, H. Ni, Activated NK cells cause placental dysfunction and miscarriages in fetal alloimmune thrombocytopenia, Nat. Commun. 8 (2017) 224, https://doi.org/10.1038/s41467-017-00269-1.
[36] K. Alhareth, L. Valero, K.E. Mohamed, L. Fliedel, C. Roques, S. Gil, N. Mignet, T. Fournier, K. Andrieux, Qualitative and quantitative analysis of the uptake of lipoplexes by villous placenta explants, Int. J. Pharm. 567 (2019) 118479, https://doi.org/10.1016/j.ijpharm.2019.118479.
[37] M. Arenas-Hernandez, E.N. Sanchez-Rodriguez, T.N. Mial, S.A. Robertson, N. Gomez-Lopez, Isolation of leukocytes from the murine tissues at the maternal-fetal interface, J. Vis. Exp. (2015) 52866, https://doi.org/10.3791/52866.
[38] G. Liang, B. Huang, F. Wang, F. Liu, Protocols for isolation and characterization of mouse placental hemogenic endothelial cells, STAR Protoc 2 (2021) 100884, https://doi.org/10.1016/j.xpro.2021.100884.
[39] B. Zhang, L. Tan, Y. Yu, B. Wang, Z. Chen, J. Han, M. Li, J. Chen, T. Xiao, B. K. Ambati, L. Cai, Q. Yang, N.R. Nayak, J. Zhang, X. Fan, Placenta-specific drug delivery by trophoblast-targeted nanoparticles in mice, Theranostics 8 (2018) 2765–2781, https://doi.org/10.7150/thno.22904.
[40] Q. Yu, Y. Qiu, X. Wang, J. Tang, Y. Liu, L. Mei, M. Li, M. Yang, L. Tang, H. Gao, Z. Zhang, W. Xu, Q. He, Efficient siRNA transfer to knockdown a placenta specific lncRNA using RGD-modified nano-liposome: a new preeclampsia-like mouse model, Int J Pharm 546 (2018) 115–124, https://doi.org/10.1016/j.ijpharm.2018.05.001.
[41] S.K. Cool, K. Breyne, E. Meyer, S.C. De Smedt, N.N. Sanders, Comparison of in vivo optical systems for bioluminescence and fluorescence imaging, J. Fluoresc. 23 (2013) 909–920, https://doi.org/10.1007/s10895-013-1215-9.
[42] J. Lei, J.M. Rosenzweig, M.K. Mishra, W. Alshehri, F. Brancusi, M. McLane, A. Almalki, R. Bahabry, H. Arif, R. Rozzah, G. Alyousif, Y. Shabi, N. Alhehaily, W. Zhong, A. Facciabene, S. Kannan, R.M. Kannan, I. Burd, Maternal dendrimer-based therapy for inflammation-induced preterm birth and perinatal brain injury, Sci. Rep. 7 (2017) 6106, https://doi.org/10.1038/s41598-017-06113-2.
[43] I.A. Vorobjev, S. Bekbayev, A. Temirgaliyev, M. Tlegenova, N.S. Barteneva, Imaging flow cytometry of multi-nuclearity, in: N.S. Barteneva, I.A. Vorobjev (Eds.), Spectral and Imaging Cytometry: Methods and Protocols, Springer US, New York, NY, 2023, pp. 87–101, https://doi.org/10.1007/978-1-0716-3020-4_5.