

Video Article

Genetic Engineering of Primary Mouse Intestinal Organoids Using Magnetic Nanoparticle Transduction Viral Vectors for Frozen Sectioning

Lingling Xian¹, Lionel Chia^{1,5}, Dan Georgess², Li Luo¹, Shuai Shuai¹, Andrew J. Ewald^{3,4}, Linda M.S. Resar^{1,4,5,6}¹Department of Medicine, Division of Hematology, Johns Hopkins University School of Medicine²Department of Natural Sciences, School of Arts & Sciences, Lebanese American University³Department of Cell Biology, Johns Hopkins University School of Medicine⁴Department of Oncology, Johns Hopkins University School of Medicine⁵Department of Pathology, Johns Hopkins University School of Medicine⁶Institute for Cell Engineering, Johns Hopkins University School of MedicineCorrespondence to: Linda M.S. Resar at lresar@jhmi.eduURL: <https://www.jove.com/video/57040>DOI: [doi:10.3791/57040](https://doi.org/10.3791/57040)

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Abstract

Intestinal organoid cultures provide a unique opportunity to investigate intestinal stem cell and crypt biology in vitro, although efficient approaches to manipulate gene expression in organoids have made limited progress in this arena. While CRISPR/Cas9 technology allows for precise genome editing of cells for organoid generation, this strategy requires extensive selection and screening by sequence analysis, which is both time-consuming and costly. Here, we provide a detailed protocol for efficient viral transduction of intestinal organoids. This approach is rapid and highly efficient, thus decreasing the time and expense inherent in CRISPR/Cas9 technology. We also present a protocol to generate frozen sections from intact organoid cultures for further analysis with immunohistochemical or immunofluorescent staining, which can be used to confirm gene expression or silencing. After successful transduction of viral vectors for gene expression or silencing is achieved, intestinal stem cell and crypt function can be rapidly assessed. Although most organoid studies employ in vitro assays, organoids can also be delivered to mice for in vivo functional analyses. Moreover, our approaches are advantageous for predicting therapeutic responses to drugs because currently available therapies generally function by modulating gene expression or protein function rather than altering the genome.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57040/>

Introduction

The ability to culture mouse or human crypts cells as three dimensional (3D) organoids from the small intestines or colon over prolonged time periods provided a major breakthrough because these cultures display defining features of intestinal epithelium in vivo^{1,2,3}. Organoids derived from primary crypts are capable of self-renewal and self-organization, exhibiting cellular functions similar to their tissues of origin. Indeed, organoids recapitulate not only the structural organization of crypts in vivo, but also many molecular features, thus providing useful tools to study normal biology and disease states. To illustrate, organoid studies have revealed novel molecular pathways involved in tissue regeneration^{1,2,3,4,5} as well as drugs that could enhance function in pathologic settings^{6,7}.

The study of intestinal stem cells is of particular interest because the intestinal lining is among the most highly regenerative mammalian tissues, renewing itself every 3-5 days to protect the organism from bacteria, toxins, and other pathogens within the intestinal lumens. Intestinal stem cells (ISCs) are responsible for this remarkable regenerative capability and thus provide a unique paradigm for studying adult stem cell function^{1,2}. Lineage-tracing experiments in mice demonstrated that isolated Lgr5-positive stem cells can be cultured to generate 3D organoids or 'mini-guts' in vitro where they closely mirror their in vivo counterparts. Organoid cultures can also be derived from intestinal crypt cell isolates comprised of progenitors, ISCs, and Paneth cells, the latter of which constitute the epithelial niche cells in vivo. In fact, organoid culture from primary intestinal crypt cells has evolved into a relatively routine technique that is easy to implement in most laboratories using widely available reagents. This model is also amenable to quantitative analysis of gene expression by RNA-sequencing (RNA-Seq) and proteins by mass spectrometry, immunohistochemistry, or immunofluorescent staining^{2,4,8}. In addition, functional genetics can be studied using gain-of-function (gene overexpression or expression of an activating mutant gene) or loss-of-function (gene silencing or expression of a loss-of-function mutant) approaches².

Importantly, low efficiency and high toxicity of standard plasmid DNA or viral transduction protocols with polybrene remain a major hurdle in the field. Although CRISPR/Cas9 technology allows for precise genome editing, this approach requires time-consuming selection followed by sequence validation⁹. Here, we present a viral transduction protocol for primary intestinal organoids that optimizes delivery of viral particles by conjugation to magnetic nanoparticles and application of a magnetic field. Key modifications to prior protocols^{4,5,10,11,12,13} and recommendations

to enhance efficiency are provided. We also describe an approach to generate frozen sections from intact organoids cultured in 3D matrigel (henceforth referred to as basement membrane matrix or matrix) for further analysis with immunohistochemistry or immunofluorescent staining.

Protocol

This protocol was approved by the Johns Hopkins Medical Institutions Animal Care and Use Committee (IACUC). This protocol is modified from a previously published methods^{10,11,12,13}.

1. Preparation of Reagents

1. Prepare fresh 293T medium several hours in advance and warm to 37 °C in a water bath for at least 10 min before use (**Table 1**).
2. Prepare plasmid DNA^{2,13,14} for viral packaging. (**Table 2**).
3. Acquire all other required materials (**Table of Materials**).

2. Lentivirus or Retrovirus Particle Production

1. **Human embryo kidney (HEK) 293T cell seeding (Day 1)**
 1. Prepare one 150 mm culture dish by coating with 50 µg/mL poly-D-lysine dissolved in phosphate buffered saline (PBS; 10 mL/dish) for 1 h at room temperature (RT).
 2. Remove the phosphate buffered saline (PBS)/poly-D-lysine and wash the coated dish twice with 5 mL PBS.
 3. Seed 293T cells (8–10 x 10⁶) in 293T medium (**Table 1**) to a total volume of 15 mL.
 4. Culture 293T cells overnight in a standard tissue culture incubator (37 °C, 5% CO₂).
2. **HEK 293T cell transfection (Day 2)**
 1. Perform transfection once cells have reached 70–80% confluence (usually about 24 h after seeding 8–10 x 10⁶ cells).
 2. Prepare transfection mixture using an efficient approach such as a commercial cationic liposome formulation (**Tables 1–2, Table of Materials**)².
 1. Dilute lentivirus DNA constructs¹² (total ~24 µg plasmid DNA, **Table 2**) in 1.2 mL of transfection medium and incubate for 5 min at RT in 1.5 mL tubes (**Table of Materials**).
 2. Dilute transfection reagent (36 µL) in 1.2 mL of transfection medium (**Table of Materials**) and incubate in 5-mL tubes for 5 min according to the manufacturer's instructions.
 3. Add the lentivirus reagent (step 2.2.2.1) to the transfection reagent (step 2.2.2.2) and gently mix by slow pipetting up and down using a 5 mL pipet.
 4. Incubate the mixture for 20 min at RT.
 3. Gently wash 293T cells with 5 mL of transfection medium and replace with 10 mL of transfection medium.
 4. Add the DNA-lipid complexes (from step 2.2.2.3) dropwise to the medium of 293T cells and carefully mix the media in the culture dishes by moving in horizontal and vertical directions to ensure equal distribution of the DNA-lipid complexes in each dish.
 5. Incubate for 6 h in a standard tissue culture incubator (37 °C, 5% CO₂).
 6. After incubation, replace the media with 20 mL fresh virus collecting medium (**Table 1**).
3. **Virus collection (Days 3–5)**
 1. Collect virus media in 50 mL tubes, and store in a 4 °C refrigerator for further concentration.
 2. Replace 20 mL of fresh virus collecting medium every 24 h and culture overnight (~24 h). Repeat media collection over the next 2 days (Days 4–5).
 3. Ensure that the total volume of medium collected after day 5 is ~ 60 mL (20 mL/day x 3 days).
4. **Virus concentration (Day 5)**
 1. Centrifuge the collected media (60 mL) for 5 min at 400 x g. Then, pass the supernatant through a filter (0.45 µm pores) to remove any cellular debris.
5. **Concentrate the virus by adding 15 mL of filtered virus media in a centrifugal filter unit (Table of Materials). Centrifuge at 2,500 x g for 15 min at 4 °C. Because the virus cannot pass through this filter, it will be concentrated in the upper chamber of the filter.**
 1. Aspirate the flow-through from the tube (bottom chamber) and add another 15 mL of remaining viral collection media supernatant to the same centrifugal filter unit. Centrifuge as above (2,500 x g for 15 min at 4 °C) to concentrate additional virus from the supernatant.
 2. Repeat the process using the same filter for 60 mL media from a single transduced plate until the desired concentration is reached (~100-fold).
Note: We typically concentrate ~60 mL of virus collection media to ~600 µL.
 3. Remove the concentrated virus from the upper chamber of the filter using a 1 mL pipet, then aliquot and store in 1.5 mL tubes (50–60 µL/tube) at -80 °C for later use. Store concentrated particles for up to 6–12 months.

3. Isolating Crypts

1. Euthanize mice using CO₂ according to the local IACUC guidelines.
2. Place the euthanized animal on its back and wash the abdomen by spraying with 70% ethanol.
3. Perform a longitudinal midline incision from the sternum to the groin, incising the skin first, and then the subcutaneous tissue.
4. Remove the small intestine from the stomach to the cecum.

5. Identify regions of interest within the small intestine; crypts can be isolated from the duodenum, jejunum and ileum.
6. Using a 10 mL syringe, flush the isolated small intestine with crypt dissociation buffer (pre-chilled PBS containing 1 mM dithiothreitol (DTT), 1% penicillin/streptomycin (no Ca^{2+} and Mg^{2+})) in a 10 cm tissue culture dish (**Table 1**)
7. Remove peripheral fat tissue, and open or "fillet" the intestinal tissue longitudinally on a sterile glass plate (15 cm x 15 cm).
8. Gently scrape off the intestinal epithelial villi using a cell scraper.
9. Cut the small intestine into 2–3 cm length-wise sections.
10. Transfer the tissue to a 15 mL tube containing pre-chilled PBS using flat forceps (116 mm). Wash the tissue fragments by shaking vigorously by hand for ~30 s (moving the tube in opposite directions ~60 times).
11. Refresh the PBS and repeat wash until the PBS becomes clear.
Note: We typically wash the fragments 2–3 times.
12. Incubate the tissue in a 15 mL conical tube containing 10 mL of crypt dissociation buffer (**Table 1**) for 10 min at 4 °C on an orbital shaker at medium speed once the PBS is clear.
13. Vigorously shake the tube by hand for ~30 s (opposite directions ~60 times) and transfer the tissue using flat forceps to another 15 mL conical tube containing 10 mL of crypt dissociation buffer. Incubate this fraction on ice. Do not use the first fraction for organoid culture because it contains primarily villi.
14. Repeat steps 3.11–3.13 for 3–4 times, collecting each fraction and placing them on ice.
15. Select the fraction that is enriched with the highest percentage of intestinal crypts by scanning 200 μL samples from each fraction under an inverted microscope (4x). Identify crypts by the typical morphology as described previously (**Figure 1A**)¹⁰.
NOTE: They will appear round or oval in shape and contain granulated Paneth cells. In contrast, villi are finger-like structures lacking the granular Paneth cells (**Figure 1B**).
16. Pass the selected fraction through a 40 μm cell strainer to obtain crypts of similar size if required. Alternatively, isolate Lgr5+ stem cells based on flow cytometry for green fluorescent protein (GFP) if mice are crossed onto the EGFP-Lgr5+ background or another suitable model that enables identification of Lgr5+ cells².
17. **Count the total number of crypts in the selected fraction as follows.**
 1. Pipette 50 μL of the selected fraction into a hemocytometer and count the number of crypts using an inverted light microscope (4x). Place ~100 crypts per well when using a 48-well plate to allow for the transduction experiments in which 3–6 wells will be transduced per experimental condition for gene silencing or overexpression.
 2. Based on the number of crypts per 50 μL , calculate the volume of crypt dissociation buffer needed and transfer that volume into a 1.5 mL tube.
Note: For example, 10 crypts per 50 μL are counted, 6 x 50 μL or 300 μL are needed for 300 crypts.
18. Centrifuge the crypts in the 1.5-mL tubes at 300 x g for 5 min.
19. Carefully discard the supernatant by gently pipetting off the upper liquid layer and resuspend the pellet in 100 μL of growth factor reduced basement membrane matrix on ice (**Table of Materials**).
20. Seed the matrix-containing crypts into a 37 °C pre-warmed 48-well plate (30 μL /well, ~100 crypts/well). Incubate the plate in a standard tissue culture incubator for 5–15 min to allow for matrix gelation (37 °C, 5% CO_2).
21. Overlay each gel with 250 μL organoid culture (ENR) medium (**Table 1**) and place the 48-well plates back into a standard tissue culture incubator (37 °C, 5% CO_2). Check the cultures for crypt organization into small, round, cystic shapes after 24 h; buds will form after 2–5 days.
22. Gently replace ENR media every 3 days. Remove old ENR media with gentle suction, taking care not touch the matrix when replacing media.
23. Passage organoid cultures every 4–7 days as previously described¹¹.

4. Organoid Fragment Preparation

1. Transduce organoids once they form (within 1–2 weeks) or after being passaged. For a single transduction experiment, prepare 2–3 wells of cultured organoids in a 48-well plate per condition with ~100–200 organoids/well or ~200–600 organoids per experimental transduction condition.
2. Exchange ENR with 250 μL transduction media (**Table 1**) and culture in the transduction media for 3 or more days or until the organoids adopt a cystic morphology. Include both Wnt3a and ROCK inhibitor (Y27632) in the transduction medium to increase the number of stem and Paneth cells; Nicotinamide (Nic) improves culture efficiency (see transduction medium in **Table 1**).
3. Mechanically rupture the dome-like basement membrane matrix structure with media and a pipet tip using a 1 mL pipet.
4. Transfer the organoids and media to a sterile 1.5 mL tube.
5. Mechanically disrupt the matrix further by pipetting with a 200 μL pipet ~10–15 times.
6. Centrifuge the organoid fragments at RT at 500 x g for 5 min.
7. Discard the supernatant carefully using a pipette and resuspend the pellet in 1 mL DMEM/F12 medium (**Table 1**).
8. Add Dispase I (6 μL at 10 mg/mL) and DNase I (2.5 μL at 10 mg/mL). Mix well by pipetting gently using a 1 mL pipet.
9. Incubate organoids at 37 °C for 20 min in the 1.5 mL tube.
10. Add 500 μL of ENR media to terminate the dissociation reaction; the serum in the ENR terminates the reaction.
11. Pass the organoid cells through a 20 μm cell strainer and centrifuge the organoid fragments at 400 x g for 5 min.
12. Resuspend organoid fragments with 150 μL transduction medium (**Table 1**).

5. Genetic Engineering of Organoids or Crypt Cells by Viral Transduction

NOTE: See **Figure 2**.

1. Seed organoid cell clusters with 200 μL transduction medium/well in a 48-well plate and incubate in a standard tissue culture incubator (37 °C, 5% CO_2). Alternatively, place freshly isolated crypt cells (~1,000 crypts) in 200 μL transduction medium/well in a 48-well plate and incubate in a standard tissue culture incubator (37 °C, 5% CO_2).
2. Thaw vials of virus for transduction allowing for ~50 μL of concentrated virus for transduction of each well in 48-well plates or ~100 μL of concentrated virus per well in 24-well plates.

3. Incubate virus with 12 μL of magnetic nanoparticle solution for 15 min at RT in a 1.5 mL tube (**Table 3**).
4. Add the magnetic nanoparticle solution/virus mixture to the cells to be transduced.
5. Place the cell culture plate on a magnetic plate and incubate for at least 2 h (and up to ~6 h) in a standard tissue culture incubator (37 °C, 5% CO_2).

6. Seeding of Infected Organoid Fragments

1. Transfer the infected organoid cell clusters and transduction media from each well into a 1.5 mL tube.
2. Centrifuge at 500 x g for 5 min.
3. Discard the supernatant with gentle suction and cool the tube containing the pellet on ice for 5 min.
4. Add 120 μL of basement membrane matrix and resuspend the pellet by pipetting slowly up and down.
5. Seed 30 μL drops of the matrix-cell mixture into a new 48-well plate.
6. Incubate the plate at 37 °C for 5–15 min until the matrix solidifies.
7. Add transduction medium to each well and incubate in a standard tissue culture incubator for 3–4 days (37 °C, 5% CO_2).
8. After 3–4 days, inspect cultures under a light microscope (10x) to ensure organization of cell clusters into organoid structures. Then, gently replace transduction media with 250 μL ENR medium.
9. Replace media every 3–4 days.

7. Selection (If Applicable)

1. After 2–3 days, add relevant antibiotics or hormones for selection to the transduction medium if appropriate.
NOTE: We used puromycin (2 $\mu\text{g}/\text{mL}$) for selection of the lentiviral transduction because plasmids harbored a puromycin resistance gene^{2,14}.

8. Confirmation of Successful Transduction and Gene Expression or Silencing

1. If using FUGW lentivirus^{2,14}, estimate transduction efficiency by measuring GFP signals via fluorescent microscope or flow cytometry.
2. Validate gene overexpression or silencing using quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for quantitative comparison of mRNA in the control and experimental organoid cultures.
3. Confirm protein levels for protein-coding gene expression or silencing by Western Blot or immunostaining².

9. Organoid Cryosection in Basement Membrane Matrix

NOTE: See **Figure 3**.

1. Remove ENR medium by gentle suction, being careful not to perturb the basement membrane matrix and gently wash once with 500 μL of PBS.
2. Fix organoids with 1.0 mL of 4% paraformaldehyde (PFA) solution (**Table of Materials**) at RT for 30 min.
3. Remove PFA by suction, and gently wash twice with 1 mL PBS.
4. Remove PBS by suction and add 1.0 mL of 30% sucrose buffer to each sample. Incubate fixed organoids in sucrose for 1 h at 4 °C in a cold room, refrigerator, or on ice to dehydrate samples.
5. Remove sucrose buffer by suction and add just enough embedding compound (**Table of Materials**) to cover the matrix layer (~300 $\mu\text{L}/\text{well}$) in each well.
6. Incubate at RT for 5 min.
7. Place samples in a -80 °C freezer for 10 min, or until the embedding compound turns solid and white.
8. Place the dish with frozen embedding compound at RT to allow for minimal melting of the compound along the edges. Use a scalpel to separate the block from the walls of the well.
9. Remove the matrix-embedding compound block using forceps and place it in a specimen block (e.g. cryomold), working quickly to prevent melting.
10. Fill the mold completely with embedding compound and freeze at -80 °C for 30 min.
11. Use the block is for sectioning or storage in -80 °C freezer for further use.

Representative Results

Here, we describe a rapid and highly efficient transduction technique which harnesses magnetic nanoparticles exposed to a magnetic field to deliver lentivirus to cells of interest. With readily available tools, we have applied this approach not only to transduce freshly isolated crypt cells (**Figure 1A**), but also for organoids (**Figure 2**) and other cells that are refractory to more routine transduction approaches. Lentiviral particles can be easily conjugated to magnetic nanoparticles and the resulting virus-nanoparticle complexes are delivered efficiently by applying a magnetic field using a magnetic plate. To optimize this approach, we first tested lentiviral vectors linked to GFP such that GFP could be used to identify transduced cells with fluorescence microscopy. The GFP can be visualized at each stage in organoid development, including early on when crypt cells organize into cyst-like structures (**Figure 4A**), or at later time points when organoids form buds (**Figure 4B**). Successfully transduced intestinal organoids can then undergo functional analysis for alterations in development by staining cell membranes and nuclei to enumerate total cell number in addition to lineage markers, such as lysozyme to identify Paneth cells (**Figure 4C**).

The genetically engineered organoids can be used for further analysis by generating frozen sections as outlined here (Figure 3). After embedding organoids, frozen blocks can be stored and later sectioned for future studies. This approach is also efficient (estimated to be ~95% based on percentage of GFP(+) organoids to total organoids). This approach can be performed with standard laboratory reagents, thus providing tissues that are amenable to diverse investigations, including cell number, cell fate, and the presence and levels of specific proteins². For example, we used frozen sections and immunofluorescent staining to identify individual cells and ascertain cell type (Figure 4C).

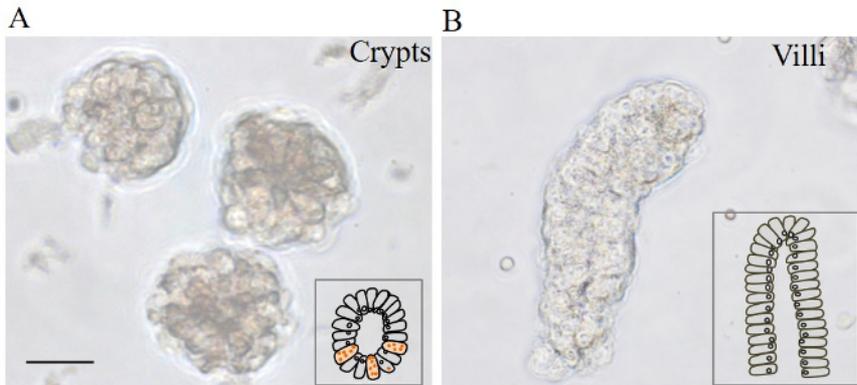


Figure 1: Isolated crypts and villi with cartoons showing typical morphology. (A) Isolated crypts form round or oval structures. (B) Villi are identified as finger-like structures. Scale bar = 50 μ m. [Please click here to view a larger version of this figure.](#)

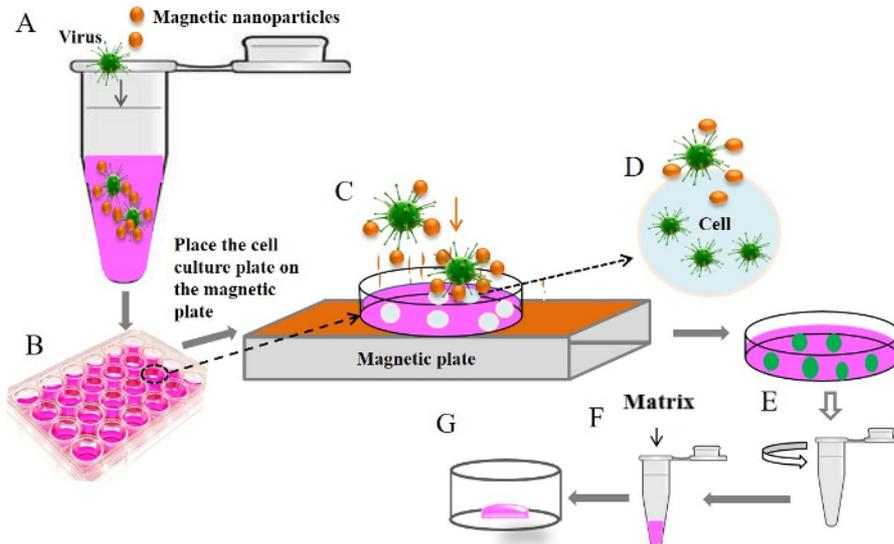


Figure 2: Schematic of viral transduction of organoids using magnetic nanoparticles and exposure to a magnetic field. The most critical steps of the transduction protocol are shown. (A) Incubate virus and magnetic nanoparticle solution for 15 min at RT in a 1.5 mL tube. (B) Add the magnetic nanoparticles/virus mixture to the cells to be transduced. (C) Place the cell culture plate on the magnetic plate and incubate for 2 h in a standard tissue culture incubator. Longer incubation times can also be used (~6 h); the representative well is shown here on the magnetic plate. (D) A cell being transduced with the virus and magnetic nanoparticle is shown. (E) Transfer the infected organoid cell clusters and transduction media from each well into a 1.5 mL tube and centrifuge at 500 x g for 5 min. Discard the supernatant with gentle suction and cool the tube containing the pellet on ice for 5 min. (F) Add 120 μ L of basement membrane matrix and resuspend the pellet by pipetting slowly up and down. (G) Seed drops of 30 μ L containing matrix-cell mixture into each well in a new 48-well plate. [Please click here to view a larger version of this figure.](#)

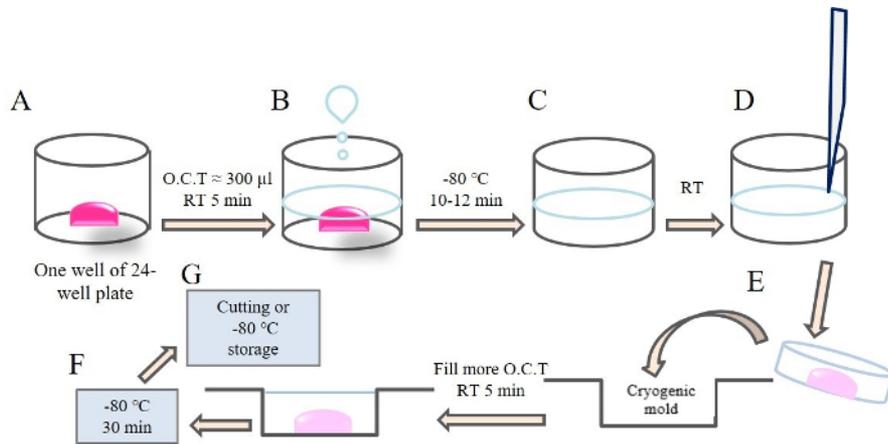


Figure 3: Schematic of frozen sectioning of organoids in 3D matrix. The most critical steps of the frozen sectioning protocol are shown. (A) A single well within a 24-well cell culture plate is depicted. (B) Add just enough embedding compound to cover the matrix layer (~300 µL/well) and incubate at RT for 5 min. (C) Place samples at -80 C in a freezer for 10 min or until the embedding compound turns solid and white. Next, place the dish at RT to allow for slight melting along the edges of the sample. (D) Use a scalpel to separate the block from the walls of the well. (E) Remove the matrix-embedding compound block using forceps and place in an appropriate shallow container or mold for freezing tissues. Fill the mold completely with embedding compound (OCT). (F) Freeze block at -80 C in a freezer for 30 min. (G) The block is ready for sectioning or storage in -80 C freezer for further use. [Please click here to view a larger version of this figure.](#)

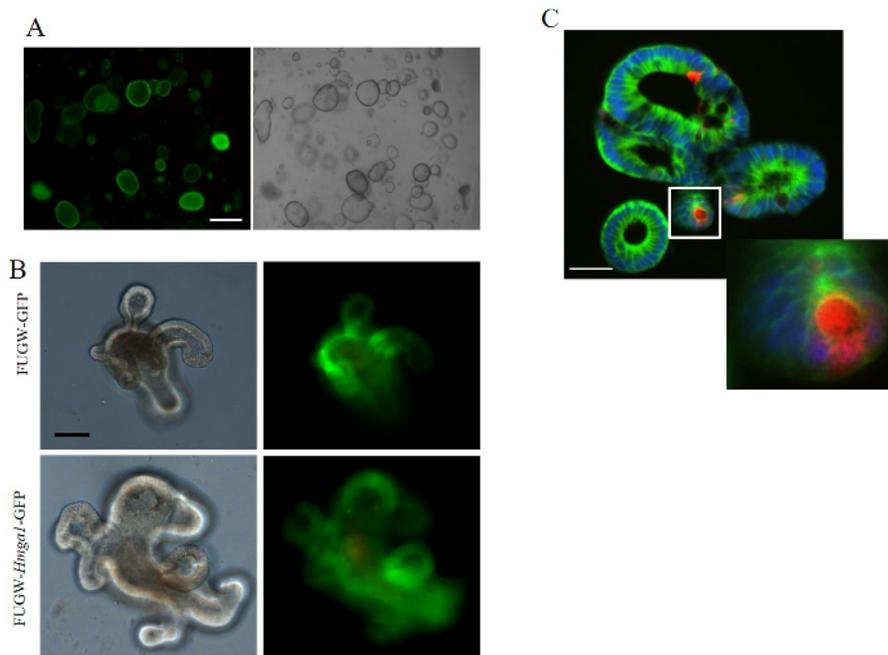


Figure 4: Representative images of transduced intestinal organoids. (A) Representative image of small intestinal organoids under light microscope showing (left) fluorescence microscopy, and, (Right) standard microscopy of transgene expression (EGFP) at day 3 after transduction. Scale bar = 50 µm. (B) Example of overexpression of gene encoding GFP in organoid after transduction using magnetic nanoparticles. Organoid cells were transduced with lentivirus expressing GFP (FUGW; Top) or lentivirus overexpressing *Hmga1* (FUGW-*Hmga1*; Bottom) as shown at day 12 after transduction. Scale bar = 50 µm. (C) Immunofluorescence imaging of formalin fixed frozen section of organoids. Organoid sections (4 µm) were stained with anti-lysozyme (red), anti-EpCAM (green) and DAPI (blue). EpCAM demarcates cell borders, DAPI indicated individual nuclei, and lysozyme stains Paneth cells. Scale bar = 50 µm. [Please click here to view a larger version of this figure.](#)

Organoid culture medium (ENR)	100 mL
DMEM/F12+	96 mL
L-alanyl-L-glutamine dipeptide supplement (e.g. Glutamax)	1 mL
NEAA	1 mL
Pen/Strep	1 mL
HEPES	1 mL
EGF (100 µg/ mL)	5 µL
Noggin (100 µg/ mL)	10 µL
R-spondin (100 µg/ mL)	10 µL
or R-spondin condition medium (CM)	20 mL
Human recombinant insulin (10 mg/ mL)	5 µL
Transduction medium	5 mL
ENR medium	2.4 mL
Wnt condition medium (CM)	2.5 mL
Nicotinamide (1M)	100 µL
Y27632 (10 µM)	10 µL
Crypts dissociation buffer	100 mL
PBS (without Ca ²⁺ , Mg ²⁺)	99 mL
Pen/Strep	1 mL
0.5 M EDTA	100 µL
0.1 M DTT (dithiothreitol)	100 µL
293T medium	100 mL
DMEM	90 mL
FBS	10 mL
Virus Collection Medium	100 mL
DMEM	99 mL
FBS	1 mL
Organoid digestion buffer	1 mL
DMEM/F12+	1 mL
Dispase I (10 mg/ mL)	6 µL
Dnase I (10 mg/ mL)	2.5 µL

Table 1: Media used in the protocol.

Plates	10 cm	15 cm
Lentivirus transducing vector	6 µg	9 µg
CMVΔR8.91	8 µg	12 µg
MD.G	2 µg	3 µg
Total vectors	≤16 µg	≤24 µg

Table 2: Quantity of plasmid DNA for transfection.

Plate	Magnetic beads (µL)	Volume of virus (µL)	Final Transduction Volume (µL)
48-well	6	50	250
24-well	12	100	500

Table 3: Volume of magnetic bead solution and vector.

Discussion

Primary culture of adult intestinal epithelium as organoids provides a powerful tool to study molecular mechanisms involved in stem cell function, intestinal epithelial homeostasis, and pathology^{1,2,3,4}. Although CRISPR/Cas9 technology can be used to genetically engineer organoids⁹, it is limited by the need for extensive screening and selection based on sequence analysis for the desired genetic changes. The goal of this protocol is to provide clear and concise instructions with video-based tutorials for magnetic nanoparticle delivery of lenti- or retrovirus to intestinal organoids, followed by frozen sectioning for further analysis.

This protocol is a rapid and efficient method to genetically engineer intestinal organoids and analyze the consequences of gene overexpression or silencing from frozen sections. Critical steps are outlined in **Figure 2**, **Figure 3**. This strategy allows for investigation of the biologic significance of genetic alterations (overexpression or silencing) in intestinal stem cells and their progeny cultured under 3D conditions^{2,13}. We have also used this magnetic nanoparticle-based delivery of viral vectors to enhance cell transduction and transgene expression in vitro in different primary cells^{2,13}.

With this approach, viral particles are coated with magnetic nanoparticles and delivered to cells by exposure to a magnetic field. Compared to current transduction methods, such as polybrene with or without spinoculation^{10,15}, magnetic nanoparticle-viral complexes are less toxic to cells because uptake of the genetic material is mediated by endocytosis and pinocytosis, two naturally-occurring biological processes that do not induce significant damage to cell membranes. Thus, both cell viability and transduction efficiency are enhanced. Transduction efficiency may be increased further using small crypt fragments or single cells (see step 4.8) instead of larger crypts or entire organoids as reported previously^{2,10,13,16}. Magnetically guided nanoparticle delivery results in rapid accumulation, penetration, and uptake of viral vectors into target cells^{2,13}. The magnetic nanoparticles are made of iron oxide, which is fully biodegradable and coated with specific proprietary cationic molecules. Nanoparticle association with viral vectors is achieved by salt-induced colloidal aggregation and electrostatic interactions. The nanoparticles are then concentrated onto cells by an external magnetic field generated by the magnetic plate placed under the culture dish. While transduction efficiency approaches 95%, not all cells are transduced, which is a limitation to this technique. In addition, endogenously expressed genes of interest are not altered as with CRISPR/Cas9 approaches.

Following gene overexpression or silencing, the organoids can be used for a myriad of studies, depending upon the scientific objectives, including analysis of gene expression, proteomic alterations within cells or secreted by cells, metabolic alterations, and morphologic changes. As with living tissues, frozen sections can be obtained for immunohistochemical and immunofluorescence studies of specific proteins such as transcription factors, cytoplasmic molecules, or cell surface markers. Our article includes an effective approach to obtain frozen sections from organoids without disturbing their position and organization in 3D culture. This is advantageous because prior techniques require the removal of the organoid from basement membrane matrix before freezing¹⁶. Processing organoids by removal from matrix could disrupt the structural organization of the organoid rather than reflect the in vitro growth and development.

This protocol to genetically engineer intestinal organoids can also be adapted to study other cell-based models and organoid systems. For example, pancreatic, colonic, hepatic, cardiac, and cerebral organoid systems could be transduced with this approach. Even cells growing under more standard culture techniques are amenable to nanoparticle technology. Furthermore, this approach can be applied to study the molecular mechanisms of diseases, not only in the context of stem cell-derived organoid systems, but also in tumor organoids.

In summary, the key modifications described in these protocols for intestinal organoid studies will hopefully empower scientists to elucidate the role of important factors and downstream pathways involved in the biology of intestinal stem cells and their progeny. These approaches should provide the means to learn more about molecular mechanisms underlying self-renewal, cell fate determination, tissue homeostasis, and intestinal epithelial regeneration, under both physiologic and pathologic conditions.

Disclosures

The authors have nothing to disclose

Acknowledgments

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