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Genetic Engineering of Intestinal Organoids via Magnetic Nanoparticle Transduction of Viral Vectors for Cryosectioning and Molecular Analysis

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JOHNS HOPKINS
MEDICINE

July 12, 2017

Dear Dr. Nandita Singh:

Thank you in advance for reviewing our manuscript for publication. Here, we describe a novel and efficient approach to genetically engineer mouse intestinal organoids. We also include our approach for cryosectioning.

Thank you in advance for the thoughtful reviews. We look forward to seeing our work in *JoVE* soon!

Best regards,

Linda Resar

TITLE:

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

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Lentivirus, Mouse, Intestinal, Organoids, Magnetic, Nanoparticles, Retrovirus

SHORT ABSTRACT:

We describe step-by-step instructions to: 1) efficiently engineer intestinal organoids using magnetic nanoparticles for lenti- or retroviral transduction, and, 2) generate frozen sections from engineered organoids. This approach provides a powerful tool to efficiently alter gene expression

in organoids for investigation of downstream effects.

LONG 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.

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*[1-3]. 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[1-5] 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) approaches[2].

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 validation[9]. 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[4,5,10-13] 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[10-13].

1. Preparation of Reagents

1.1. 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**).

1.2. Prepare plasmid DNA[2,13,14] for viral packaging. (**Table 2**).

1.3. Acquire all other required materials (**Table of Materials**).

2. Lentivirus or retrovirus particle production

2.1. Human embryo kidney (HEK) 293T cell seeding (Day 1)

2.1.1. 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).

2.1.2. Remove the phosphate buffered saline (PBS)/poly-D-lysine and wash the coated dish twice with 5 mL PBS.

2.1.3. Seed 293T cells (8–10 x 10^6) in 293T medium (**Table 1**) to a total volume of 15 mL.

2.1.4. Culture 293T cells overnight in a standard tissue culture incubator (37 °C, 5% CO_2).

2.2. HEK 293T cell transfection (Day 2)

2.2.1. Perform transfection once cells have reached 70-80% confluence (usually about 24 h after seeding 8–10 x 10^6 cells).

2.2.2. Prepare transfection mixture using an efficient approach such as a commercial cationic liposome formulation (**Tables 1–2**, **Table of Materials**)[2].

2.2.2.1. Dilute lentivirus DNA constructs[12] (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**).

2.2.2.2. Dilute transfection regent (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.

2.2.2.3. 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.

2.2.2.4. Incubate the mixture for 20 min at RT.

2.2.3. Gently wash 293T cells with 5 mL of transfection medium and replace with 10 mL of transfection medium.

2.2.4. Add the DNA-lipid complexes (from step 2.2.23) 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.

2.2.5. Incubate for 6 h in a standard tissue culture incubator (37 °C, 5% CO_2).

2.2.6. After incubation, replace the media with 20 mL fresh virus collecting medium (**Table 1**).

2.3. Virus collection (Days 3–5)

2.3.1. Collect virus media in 50-mL tubes, and store in a 4 °C refrigerator for further concentration.

2.3.2. 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).

2.3.3. Ensure that the total volume of medium collected after day 5 is ~ 60 mL (20 mL/day x 3 days).

2.4. Virus concentration (Day 5)

2.4.1. 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.

2.5. Concentrate the virus by adding 15 mL of filtered virus media in a centrifugal filter unit (**Table of Materials**). Centrifuge at 2500 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.

2.5.1. 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 (2500 x g for 15 min at 4 °C) to concentrate additional virus from the supernatant.

2.5.2. 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.

2.5.3. 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

3.1. Euthanize mice using CO_2 according to the local IACUC guidelines.

3.2. Place the euthanized animal on its back and wash the abdomen by spraying with 70% ethanol.

3.3. Perform a longitudinal midline incision from the sternum to the groin, incising the skin first, and then the subcutaneous tissue.

3.4. Remove the small intestine from the stomach to the cecum.

3.5. Identify regions of interest within the small intestine; crypts can be isolated from the duodenum, jejunum and ileum.

3.6. 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**)

3.7. Remove peripheral fat tissue, and open or "fillet" the intestinal tissue longitudinally on a sterile glass plate (15 cm x 15 cm).

3.8. Gently scrape off the intestinal epithelial villi using a cell scraper.

3.9. Cut the small intestine into 2–3 cm length-wise sections.

3.10. 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).

3.11. Refresh the PBS and repeat wash until the PBS becomes clear.

Note: We typically wash the fragments 2–3 times.

3.12. 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.

3.13. 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.

3.14. Repeat steps 3.11–3.13 for 3–4 times, collecting each fraction and placing them on ice.

3.15. 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**).

3.16. 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+ cells[2].

3.17. Count the total number of crypts in the selected fraction as follows.

3.17.1. 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.

3.17.2. 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.

3.18. Centrifuge the crypts in the 1.5-mL tubes at 300 x g for 5 min.

3.19. 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**).

3.20. 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).

3.21. 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.

3.22. Gently replace ENR media every 3 days. Remove old ENR media with gentle suction, taking care not touch the matrix when replacing media.

3.23. Passage organoid cultures every 4–7 days as previously described[11].

4. Organoid fragment preparation

4.1. 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.

4.2. 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**).

4.3. Mechanically rupture the dome-like basement membrane matrix structure with media and a pipet tip using a 1-mL pipet.

4.4. Transfer the organoids and media to a sterile 1.5-mL tube.

4.5. Mechanically disrupt the matrix further by pipetting with a 200-µL pipet ~ 10–15 times.

4.6. Centrifuge the organoid fragments at RT at 500 x g for 5 min.

4.7. Discard the supernatant carefully using a pipette and resuspend the pellet in 1 mL DMEM/F12 medium (**Table 1**).

4.8. 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.

4.9. Incubate organoids at 37 °C for 20 min in the 1.5-mL tube.

4.10. Add 500 µL of ENR media to terminate the dissociation reaction; the serum in the ENR terminates the reaction.

4.11. Pass the organoid cells through a 20-µm cell strainer and centrifuge the organoid fragments at 400 x g for 5 min.

4.12. 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**.

5.1. 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 (~ 1000 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).

5.2. 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.

5.3. Incubate virus with 12 µL of magnetic nanoparticle solution for 15 min at RT in a 1.5-mL tube (**Table 3**).

5.4. Add the magnetic nanoparticle solution/virus mixture to the cells to be transduced.

5.5. 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

6.1. Transfer the infected organoid cell clusters and transduction media from each well into a 1.5-mL tube.

6.2. Centrifuge at 500 x g for 5 min.

6.3. Discard the supernatant with gentle suction and cool the tube containing the pellet on ice for 5 min.

6.4. Add 120 µL of basement membrane matrix and resuspend the pellet by pipetting slowly up and down.

6.5. Seed 30 µL drops of the matrix-cell mixture into a new 48-well plate.

6.6. Incubate the plate at 37 °C for 5–15 min until the matrix solidifies.

6.7. Add transduction medium to each well and incubate in a standard tissue culture incubator for 3–4 days (37 °C, 5% CO_2).

6.8. 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.

6.9. Replace media every 3–4 days.

7. Selection (if applicable)

7.1. After 2–3 days, add relevant antibiotics or hormones for selection to the transduction medium if appropriate.

Note: We used puromycin (2 µg/mL) for selection of the lentivrial transduction because plasmids harbored a puromycin resistance gene[2,14].

8. Confirmation of successful transduction and gene expression or silencing

8.1. If using FUGW lentivirus[2,14], estimate transduction efficiency by measuring GFP signals via fluorescent microscope or flow cytometry.

8.2. 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.

8.3. Confirm protein levels for protein-coding gene expression or silencing by Western Blot or immunostaining[2].

9. Organoid cryosection in basement membrane matrix

Note: See **Figure 3.**

9.1. Remove ENR medium by gentle suction, being careful not to perturb the basement membrane matrix and gently wash once with 500 µL of PBS.

9.2. Fix organoids with 1.0 mL of 4% paraformaldehyde (PFA) solution (**Table of Materials**) at RT for 30 min.

9.3. Remove PFA by suction, and gently wash twice with 1 mL PBS.

9.4. 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.

9.5. Remove sucrose buffer by suction and add just enough embedding compound (**Table of Materials**) to cover the matrix layer (~300 µL/well) in each well.

9.6. Incubate at RT for 5 min.

9.7. Place samples in a -80 °C freezer for 10 min, or until the embedding compound turns solid and white.

9.8. 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.

9.9. Remove the matrix-embedding compound block using forceps and place it in a specimen block (*e.g.* cryomold), working quickly to prevent melting.

9.10. Fill the mold completely with embedding compound and freeze at -80 °C for 30 min.

9.11. 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 proteins[2]. For example, we used frozen sections and immunofluorescent staining to identify individual cells and ascertain cell type (**Figure 4C**).

FIGURE LEGENDS:

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 µm.

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 µL of basement membrane matrix and resuspend the pellet by pipetting slowly up and down. **(G)** Seed drops of 30 µL containing matrix-cell mixture into each well in a new 48-well plate.

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.

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.

Table 1. Media used in the protocol.

Table 2. Quantity of plasmid DNA for transfection.

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[1-4]. Although CRISPR/Cas9 technology can be used to genetically engineer organoids[9], 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 **Figures 2**–**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 freezing[16]. 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.

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DISCLOSURES:

The authors have nothing to disclose

REFERENCES:

1. Cheung EC, et al. TIGAR is required for efficient intestinal regeneration and tumorigenesis. *Dev Cell*. **25**(5), 463-77, doi: 10.1016/j.devcel.2013.05.001 (2013).

2. Xian L, *et al*. Hmga1 amplifies Wnt Signaling and expands the intestinal stem cell compartment and Paneth cell niche. *Nature Comm.* doi: 10.1038/ncomms15008 (2017).

3. Sato, T. *et al*. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*. **459**(7244), 262-265, doi:10.1038/nature07935 (2009).

4. Koo, B. K. *et al.* Controlled gene expression in primary Lgr5 organoid cultures. *Nature Methods.* **9**(1), 81-83, doi: 10.1038/nmeth.1802 (2012).

5. Kabiri, Z. *et al*. Stroma provides an intestinal stem cell niche in the absence of epithelial Wnts. *Development* **141**, 2206–2215 (2014).

6. Boj SF, *et al*. Organoid models of human and mouse ductal pancreatic cancer. *Ce*ll. **160** (1-2):324-38. doi:10.1016/j.cell.2014.12.021(2015)

7. Boj SF, *et al*. Forskolin-induced Swelling in Intestinal Organoids: An In Vitro Assay for Assessing Drug Response in Cystic Fibrosis Patients. *J Vis Exp*. **11** (120), doi: 10.3791/55159 (2017).

8. Muñoz J., et al. The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent '+4' cellmarkers. *EMBO J*. **31** (14): 3079-91. doi: 10.1038/emboj.2012.166 (2012).

9. Schwank G., *et al*. Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell.* **13** (6):653-8. doi: 10.1016/j.stem.2013.11.002 (2016)

10. Andersson-Rolf A, Fink J, Mustata RC, Koo BK. A video protocol of retroviral infection in primary intestinal organoid culture. *J Vis Exp.* **11** (90): e51765. doi: 10.3791/51765 (2014)

11. Sato T and Clevers H. Primary mouse small intestinal epithelial cell cultures. *Methods Mol Biol*. **945**,319-28. doi: 10.1007/978-1-62703-125-7_19 (2013).

12. Ye Z, Yu X and Cheng L. Lentiviral gene transduction of mouse and human stem cells. *Methods Mol Biol.* **430**, 243-53. doi: 10.1007/978-1-59745-182-6_17 (2008).

13. Cheung KJ, Gabrielson E, Werb Z, Ewald AJ. Collective invasion in breast cancer requires a conserved basal epithelial program. *Cell*. **155** (7):1639-51, doi: 10.1016/j.cell.2013.11.029 (2013).

14. Tesfaye A, et al. The High-Mobility Group A1 gene up-regulates *Cyclooxygenase-2* expression in uterine tumorigenesis. *Cancer Res* **67** (9)**,** 3998-04. Doi: 10.1158/0008-5472 (2007).

15. Haim, H., *et al*. Synchronized infection of cell cultures by magnetically controlled virus. *J. Virol*. **79** (1), 622-5. doi: 10.1128/JVI.79.1.622-625 (2005).

16. Xue X, Shah YM. In vitro organoid culture of primary mouse colon tumors. *J Vis Exp.* **17** (75): e50210. doi: 10.3791/50210 (2013).

Figure 1

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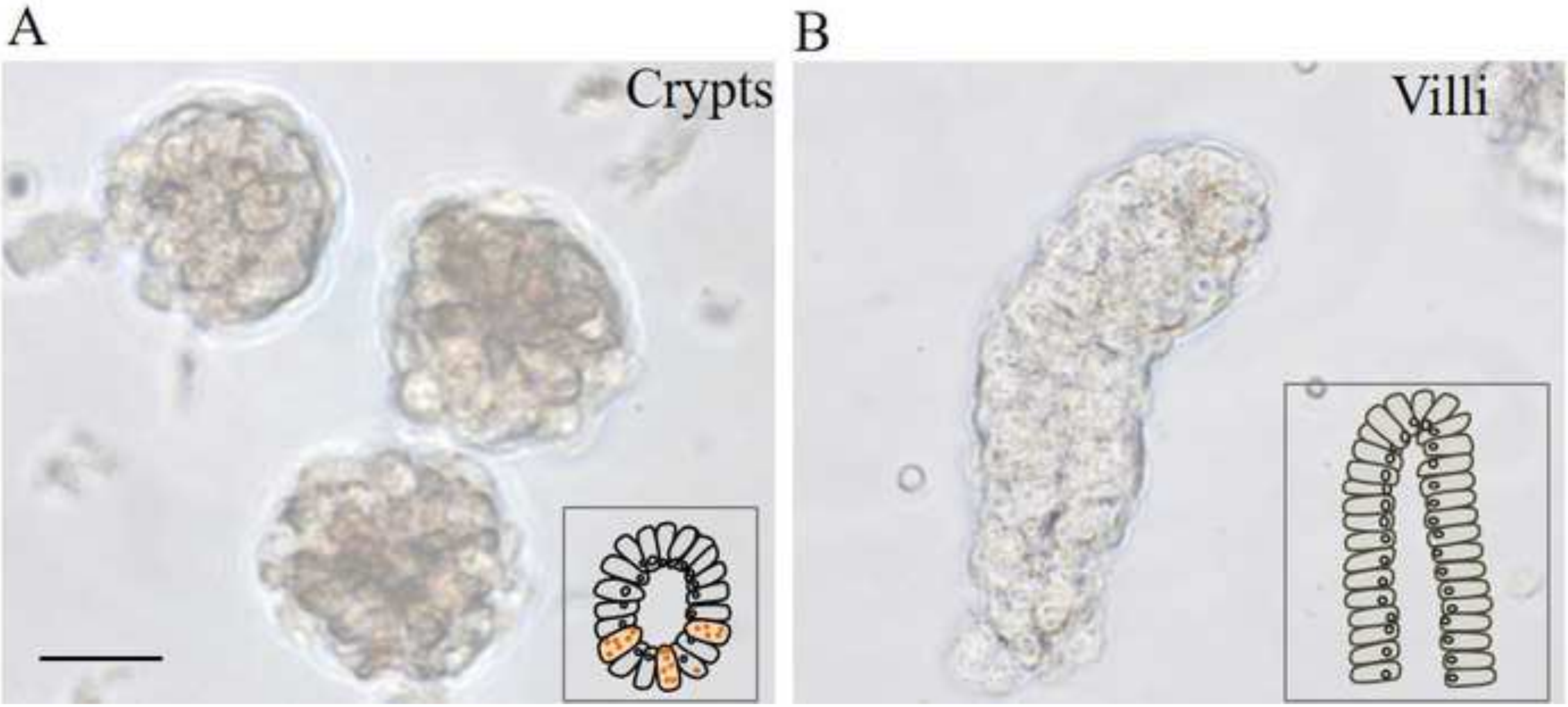

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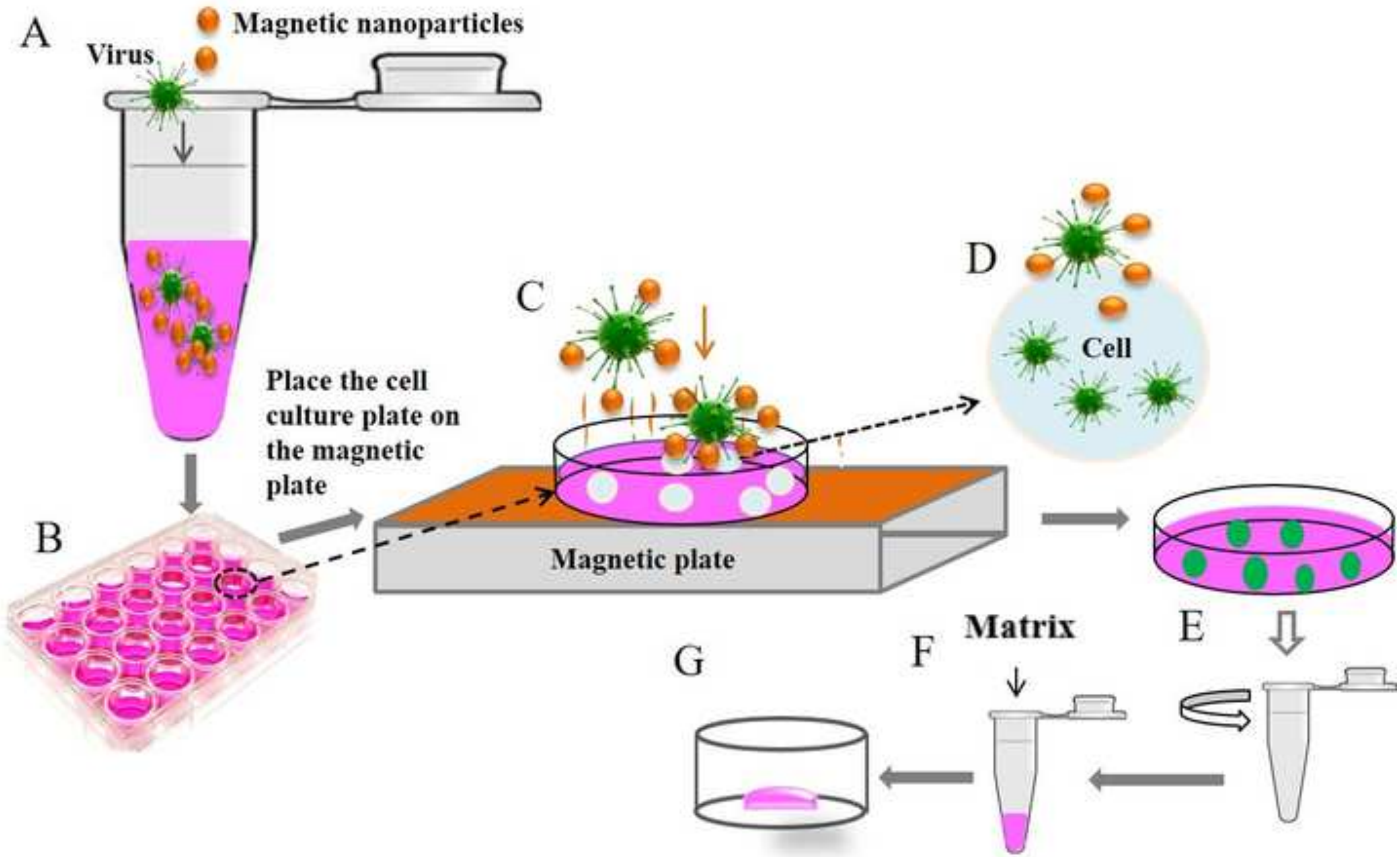

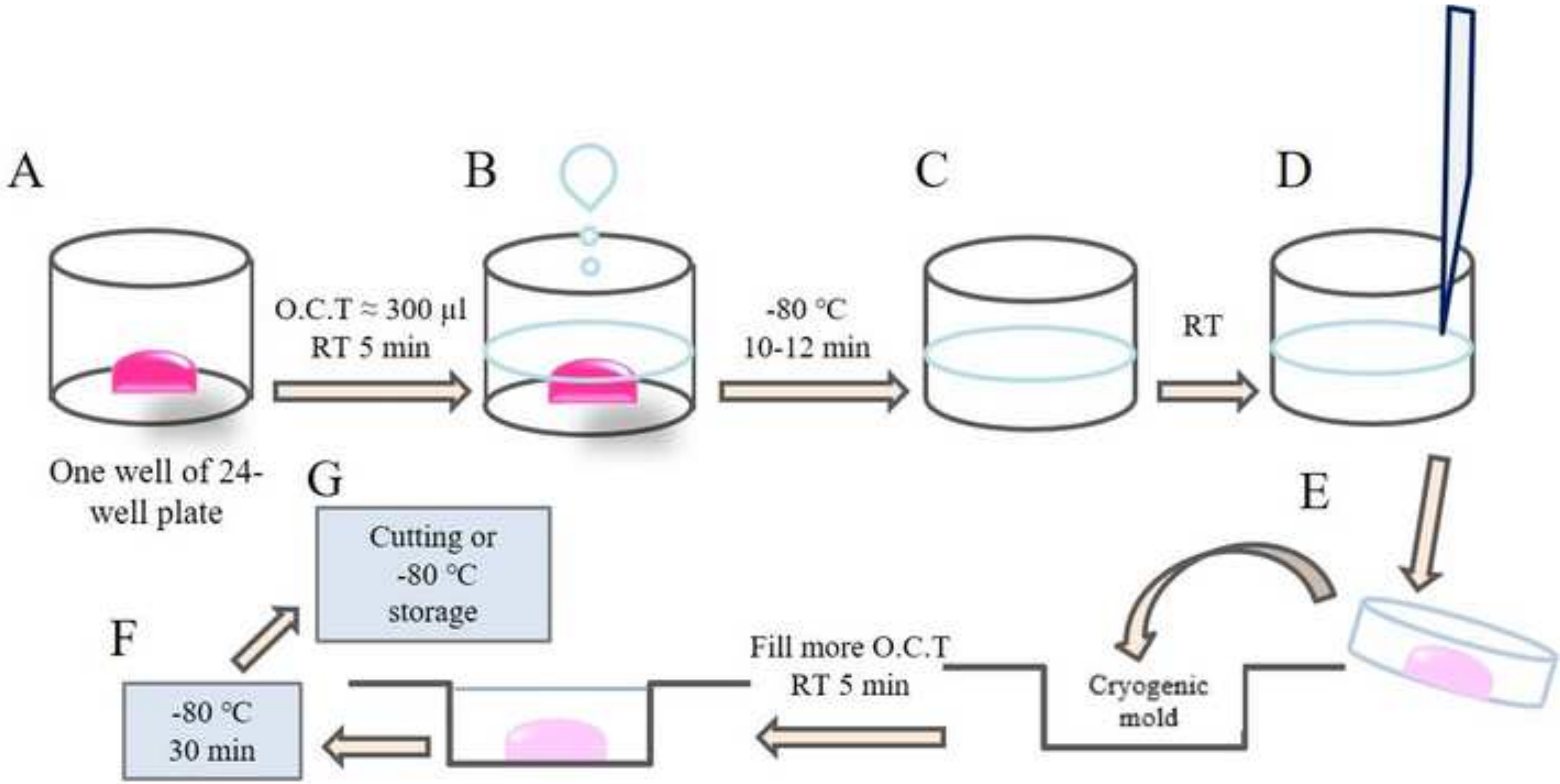
A
B
C
D
O.C.T ≈ 300 µl
RT 5 min
-80 °C
10-12 min
RT
One well of 24-well plate
G
Cutting or
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storage
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30 min
Fill more O.C.T
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Cryogenic
mold

Figure 4

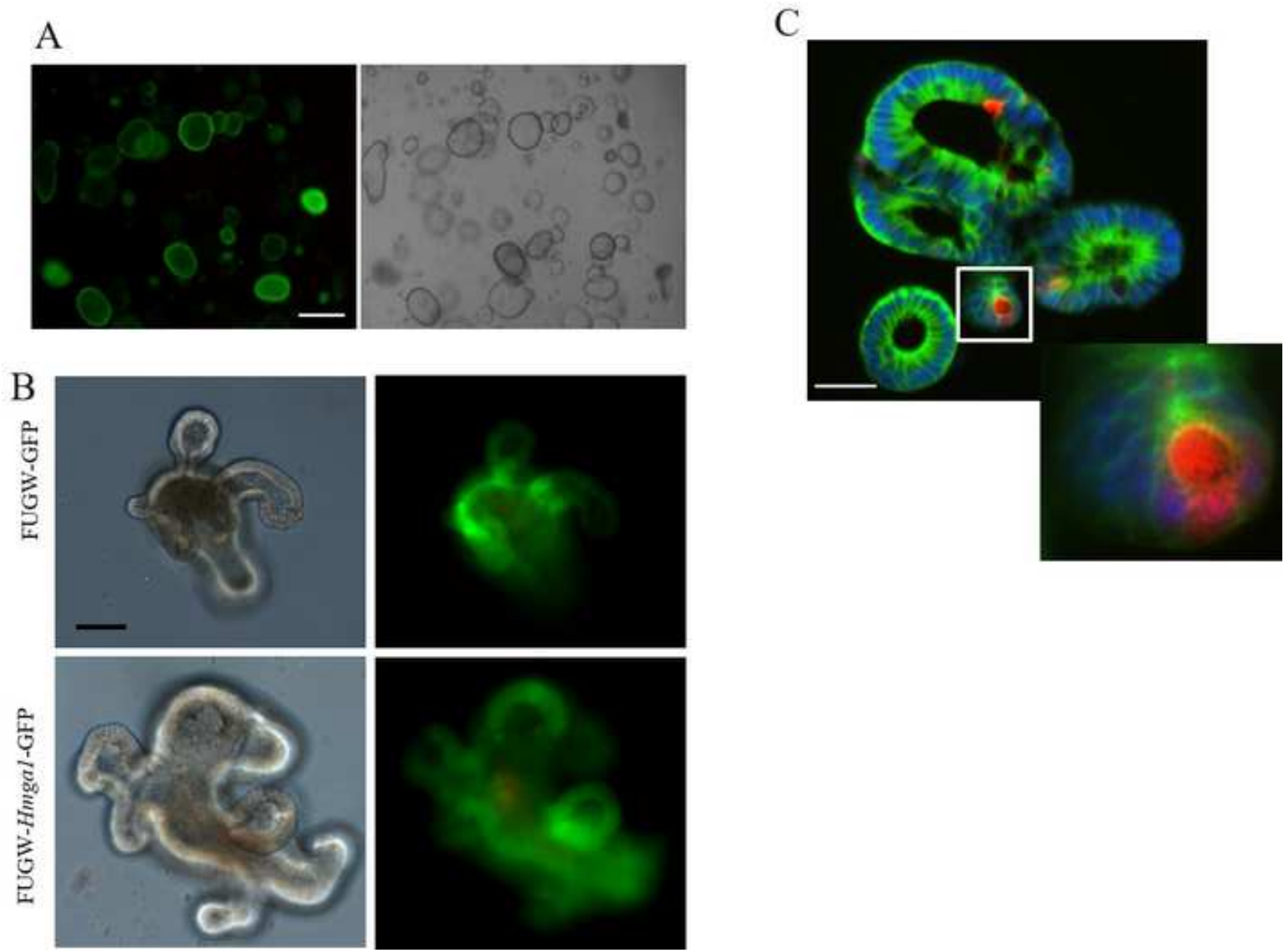

Table 1

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) or R-spondin condition medium (CM)	10 µL 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
0.1M 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

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

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

Name of Material/ Equipment	Company	Catalog Number
DMEM	Thermo Fisher Scientific	11965092
DMEM/F12+	Thermo Fisher Scientific	12634010
OPTI-MEM	Thermo Fisher Scientific	11058021
Fetal Bovine Serum	Corning	35-011-CV
Pen/Strep	Thermo Fisher Scientific	15140122
PBS (without Ca^{2+}, Mg^{2+})	Thermo Fisher Scientific	10010049
Mem-NEAA	Thermo Fisher Scientific	11140050
GlutamaxII	Thermo Fisher Scientific	35050061
HEPES	Thermo Fisher Scientific	15630080
EGF	Millipore Sigma	E9644
Noggin	Peprotech	250-38 B
R-spondin	R&D	7150-RS-025/CF
Human recombinant insulin	Millipore Sigma	I9278-5ml
Nicotinamide	Millipore Sigma	N3376-100G
Wnt3A	R&D	5036-WN-010
Y27632	Millipore Sigma	Y0503-1MG
0.5M EDTA	Thermo Fisher Scientific	15575020
DTT (dithiothreitol)	Thermo Fisher Scientific	R0861
Dispase I	Millipore Sigma	D4818-2MG
DNase I	Millipore Sigma	11284932001
matrigel(Growth factor reduced)	Corning	356231
Opti-MEM	Thermo Fisher Scientific	31985070
ViralMag R/L	Oz Biosiences	RL40200
Magnetic plate	Oz Biosiences	MF10000
Lipofectamine 2000	Thermo Fisher Scientific	11668019
Poly-D-Lysine	Millipore Sigma	A-003-E
4% Formaldehyde Solution	Boster	AR1068
O.C.T embedding compound	Thermo Fisher Scientific	4583S
5 mL Falcon polystyrene tubes	Corning	352054

50 mL Falcon Tubes	Sarstedt	62.547.100
Orbitron rotator II Rocker Shaker	Boekel Scientific	260250
Olympus Inverted microscop CK30	Olympus	CK30
Zeiss Axiovert 200 inverted fluorescence	Nikon	Axiovert 200
Amicon Ultra-15 Centrifugal Filter unit with Ultracel-100 me	Milipore Sigma	UFC910024
pluriStrainer 20 µm (Cell Strainer)	pluriSelect	SKU 43-50020
Falcon Cell Strainer	Fisher Scientific	352340
Greiner CELLSTAR multiwell culture plates 48 wells (TC tre	Millipore Sigma	M8937-100EA
Animal strain: C57BL/6J	Jackson Lab	#000664

Comments/Description
Base medium for 293T cells
Base medium for organoid culture medium and organoid digestion buffer
Virus plasmids transfection medium
Component of virus collection medium and 293T medium
Component of organoid culture medium and crypt dissociation buffer
A wash buffer and component of crypt dissociation buffer
Component of organoid culture medium
Component of organoid culture medium
Component of organoid culture medium
Component of organoid culture medium
Component of organoid culture medium
Component of organoid culture medium
Component of organoid culture medium
Component of Transduction medium
Component of Transduction medium
Component of Transduction medium
Component of Crypts dissociation buffer
Component of Crypts dissociation buffer
Component of organoid digestion buffer
Component of organoid digestion buffer
Used as a matrix to embed organoids
Medium for transfection in viral production
Magnetic particles of viral transduction
Magnetic plate to facilitate viral transduction
Transfection agent in viral production
Coating for plates before seeding 293T cells
Solution to fix organoids
For embedding of the the organoids

for scanning and counting crypts
for viewing fluorescence in the crypts
For concentrating viruses
For preparing organoid fragments
For preparing cyrpts of similar size after crypt isolation
ForD2:D37+D16:D37g organoid fragments
For organoid culture

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Author(s): Lingling Xian, Lionel Chia, Dan Georgess, Li Luo, Shuai Shuai, Andrew J. Ewald, and Linda M. S. Resar

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Dear Dr. Dsouza:

We would like to thank the reviewers for the thoughtful review and constructive comments regarding our manuscript. Our response to each reviewer and editor is detailed below in a point-by-point fashion; our changes to the manuscript are highlighted in blue.

Comments from Peer-Reviewers:
Reviewer #1:
Manuscript Summary:
Authors have elegantly presented a viral transduction as alternative and efficient method for the gene modification. They also described a protocol to generate frozen sections from intact organoids for IHC.
- We appreciate this reviewer's positive comments.

Reviewer #2:
Manuscript Summary:
The manuscript by Xian and colleagues provides a detailed protocol to modify gene expression in intestinal organoids in 3D cultures. The method is based upon viral transduction coupled with magnetic nanoparticles and application of a magnetic field to increase transduction efficiency. Intestinal 3D cultures are currently considered as a major tool to study intestinal stem cells in an integrated context. Several protocols have described methods to modify gene expression in organoids, but their efficiency remains low. CRISPR/Cas9-based genome editing has also been proposed as a key tool to engineer isolated intestinal stem cells - essentially to introduce point mutations-, but transfection of these cells remains a challenging issue. Thus, this new technology of viral transduction, nanoparticles and magnetic field appears of great interest when considering altering the expression of a gene by gain-of-function/loss-of-function approaches.
In summary, the article is clear, well written and more importantly, the methodology excellently detailed.
I have no further comments.
--We appreciate this reviewer's positive comments

Reviewer #3:
Manuscript Summary:
The manuscript describes a protocol for viral-mediated transduction of organoids in order to genetically manipulate their DNA. This protocol is well written and very easy to follow. More importantly, it will allow other laboratories to use this technique for their own in vitro studies of intestinal organoids.
We appreciate this reviewer's positive comments.

Major Concerns:
No major concerns

Minor Concerns:

It might be a good idea to show original pictures as well as a cartoon of crypt and villus
The GFP in Figure 4B is slightly out of focus.
- The GFP in Figure 4B appears slightly out of focus because GFP is expressed in a 3D plane whereas the image is taken as a 2D plane. Thus, some GFP that will be out of focus.

Reviewer #4:
Manuscript Summary:
In the manuscript "Genetic Engineering of Intestinal Organoids via Magnetic Nanoparticle Transduction of Viral Vectors for Cryosectioning and Molecular Analysis", the authors describe the process of preparing for and culturing intestinal crypts for transduction and downstream applications. They do a thorough job of describing the techniques used and present a clear protocol for others to follow. If the authors could address the following very minor issues, the manuscript would be of interest to JoVE.

Major Concerns:
None

Minor Concerns:
1. For how long can the concentrated viral particles be stored at -80C? (Section 2.4 step 5)
- Concentrated particles can be stored for 6 months to a year. We added this to the text at 2.4.5 to clarify this important point.
2. What is the recommended timeframe between steps 3 and 4?
- Transductions can be performed directly on isolated crypt cells prior to forming organoids or once organoids have been established and passaged. While we have not formally tested whether the time that organoids are cultured affects the transduction efficiency, we have performed transductions in organoids generated after several days or several weeks. We added a new step (4.1) to clarify this important point. We also edited step 5.1 to indicate that isolated crypt cells can also be transduced.
3. For step 8, are cultures disrupted in the same manner as used for viral transduction?
- Step 8 does not require disruption of cultures.
4. Figure 1a and 1b would be clearer with labels for the cells or a short description. Table 1 is hard to follow.
-We appreciate the suggestion and added labels as well as a short description with images of crypts and villi. Table 1 was also been revised.

<u>Response to the JoVE Editors</u>
Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
1) Please ensure that all text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
a) Examples NOT imperative tense: “To isolate crypts from the small intestine, mice are first humanely sacrificed according...”;” Approximately 24 h after seeding, the crypts will organize into small, round cystic shapes”; Lines 225-227 etc.

- The text was revised to the imperative tense.

• Please ensure that the manuscript title best reflects the filmable content (i.e. the portions you highlight).
-Yes, the title reflects the filmable content.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more details to the following protocol steps (please note that this is guide, and not a complete list). Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
1) Line 113: Please cite a reference for the plasmid use.
- We now cite a references for the plasmid used.
2) Line 142: Mention culture temperature and duration.
- Culture temperature and duration were added.
3) Line 167: Mention euthanasia method.
- Euthanasia method was added.
4) Line 170: Mention dissection tools used.
- Dissection tools were added.
5) Line 259: Mention incubation temperature.
- Incubation temperature was added.
6) Section 8: More details are needed for fluorescence microscope, and flow cytometry, RT PCR (mention primers, and cycle conditions).
- Section 8 is a validation step for gene expression and/or protein levels. Therefore, investigators should use their own optimized protocols to validate their gene or protein of interest.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.
- The protocol has been formatted accordingly.

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The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
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Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
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The protocol has been formatted accordingly.

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- A paragraph of the results text was added to the Representative Results section.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
The discussion was modified to include the following:
1) Modification and troubleshooting of the protocol
2) Limitations
3) Significance of methods with respect to existing methods
4) Additional future applications
5) Critical steps

• **Figure/Tables:**
1) Fig 1, Fig 4A, B, C: Please expand the legends to adequately describe the figures, e.g. what do the colors indicate? Please discuss the significance.
2) Fig 4A, C: Please provide scale bars, and define them in the figure legend.
We have addressed these issues.

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- Commercial names were eliminated but could be found by checking the indicated references or Materials Tables if needed.
2) Please check Table 1, and Figure 2 as well

- Corrected as noted above.

• **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as animals strains, microscope, etc
- Table of materials have been updated accordingly.

• Please define all abbreviations at first use (e.g. DTT, ENR, etc)
- Abbreviations were defined at first use.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.
- All SI units have been formatted.

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