Video Article

A Protocol for Lentiviral Transduction and Downstream Analysis of Intestinal Organoids

Jooske F. Van Lidth de Jeude¹, Jacqueline L. M. Vermeulen¹, Paula S. Montenegro-Miranda¹, Gijs R. Van den Brink¹, Jarom Heijmans¹

¹Tytgat Institute for Liver and Intestinal Research, Department of Gastroenterology and Hepatology, Academic Medical Center

Correspondence to: Jarom Heijmans at j.heijmans@amc.nl

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Abstract

Intestinal crypt-villus structures termed *organoids*, can be kept in sustained culture three dimensionally when supplemented with the appropriate growth factors. Since organoids are highly similar to the original tissue in terms of homeostatic stem cell differentiation, cell polarity and presence of all terminally differentiated cell types known to the adult intestinal epithelium, they serve as an essential resource in experimental research on the epithelium. The possibility to express transgenes or interfering RNA using lentiviral or retroviral vectors in organoids has increased opportunities for functional analysis of the intestinal epithelium and intestinal stem cells, surpassing traditional mouse transgenics in speed and cost. In the current video protocol we show how to utilize transduction of small intestinal organoids with lentiviral vectors illustrated by use of doxycyclin inducible transgenes, or IPTG inducible short hairpin RNA for overexpression or gene knockdown. Furthermore, considering organoid culture yields minute cell counts that may even be reduced by experimental treatment, we explain how to process organoids for downstream analysis aimed at quantitative RT-PCR, RNA-microarray and immunohistochemistry. Techniques that enable transgene expression and gene knock down in intestinal organoids contribute to the research potential that these intestinal epithelial structures hold, establishing organoid culture as a new standard in cell culture.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52531/>

Introduction

The intestinal epithelium is one of the most rapidly proliferating bodily tissues, which has caused it to attract wide interest from research on cancer and stem cells. In 2009 a technique was published to generate long lasting cultures of small intestinal crypts in matrigel, conserving a 3 dimensional structure¹. These structures, termed intestinal organoids, can be cultured using standard techniques, with surrounding medium supplemented with a number of defined growth factors, including the Bmp-signaling pathway inhibitor noggin (Nog), the Wnt-signaling pathway enhancer rspondin 1 (Rspo1) and epidermal growth factor (Egf) all found to enhance intestinal proliferation²⁻⁴.

Organoids surpass traditional cancer cell lines in the aspects that they are non-mutated, have maintained stem cell hierarchy, display intact cellular polarization and exhibit differentiation into all cell lineages found in the nascent small intestinal epithelium. Since they can be transduced to carry transgenes or RNA interference constructs⁵, they are used to study specific genetic elements, outweighing experiments using transgenic mice in facets of cost and speed. Transgenic expression in organoids can be performed using either murine retroviral or lentiviral vectors^{6,7}. Due to the limitations of murine retroviruses, capable of transducing mitotic cells exclusively⁸, lentiviral transduction is more frequently used for cells that are difficult to infect, such as organoids.

Virally transduced and stably expressing transgenic organoids can be used for a multitude of downstream analyses, including quantitative RNA analyses and immunohistochemistry. Taken together, culture of organoids from primary intestinal epithelial cells has evolved into a routine technique that is easy to implement without specific laboratory requirements, and has become the novel standard in cell culture in research on the intestinal epithelium.

Techniques of viral transduction and subsequent downstream analysis in organoids are tedious to perform and to aid organoid experiments we generated this video protocol, showing methods for lentiviral transduction of cultured organoids. We additionally show how correct processing of organoids can increase yield and therefore enhance performance of downstream analysis using RNA techniques or immunohistochemistry. In the protocol, organoids that are derived from small intestinal crypts were exclusively used, although the techniques described may be applied to colonic organoids as well.

Protocol

1. Preparation of Polyethylenimine (PEI) as Transfection Reagent

1. Dissolve approximately 150 mg of PEI into 100 ml of H₂O.
2. Adjust solution to pH 7.4 by adding HCl until solution becomes clear and stir until completely dissolved. This may take between 10 and 60 min and add water to an end concentration of 1 mg/ml.
3. When clear, filter the PEI solution through sterile 0.22 μ m filter and store in a -80 °C freezer in aliquots of 5 ml.

2. Production of Lentiviral Particles

Day 1:

1. Split HEK293T cells to 60%-80% confluency in 162 cm² flask or large petri dish in cell line culture medium (DMEM supplemented with 10% FCS, 1% penicillin/streptomycin and 2 mM glutamine supplement).

Day 2:

2. Prepare DNA transfection solution containing 45 μ g of total plasmid DNA by adding together lentiviral packaging vectors (7 μ g of pVSVg; 5 μ g of pRSV rev; 13 μ g of pMDL) and 20 μ g of lentiviral plasmid encoding the gene of interest or shRNA of interest. Adjust to a volume of 1 ml using DMEM.
3. Prepare PEI transfection solution by adding 90 μ l of 1 mg/ml PEI to 930 μ l of DMEM and incubate for 5 min at room temperature.
4. Add DNA transfection solution to PEI solution. Vortex or invert a number of times and incubate for 5 min at room temperature to obtain DNA transfection solution.
5. Drip 2 ml of the DNA transfection solution onto the HEK293T cells and incubate for 4 hr in a humidified cell culture incubator on 37 °C.
6. After 4 hr, refresh the culture medium to remove PEI. It is not necessary to wash cells before adding new medium.
NOTE: PEI is cytotoxic and incubation times longer than 4 hr may cause harm to the HEK293T cells.

Day 4:

7. Replace supernatant with new culture medium. Keep supernatant (containing virus); this will be used in step 2.10.
8. Put supernatant in 15 ml flask. To remove dead cells, centrifuge for 5 min at 500 x g.
9. Push supernatant through 0.45 μ m filter using a large 60 ml syringe. Store overnight at 4 °C.

Day 5:

10. Collect the second batch of supernatant; centrifuge and filter as in steps 2.8-2.9.
11. Pool the supernatants from steps 2.9 and 2.10 in ultracentrifuge tubes and centrifuge at 50,000 x g in an ultracentrifuge for 90 min.
12. Take out capsules containing the ultracentrifuge tubes very carefully and put into a laminar flow hood, remembering the orientation of the tube inside the centrifuge.
13. Open capsule holding ultracentrifuge tube and decant medium carefully in such a fashion that the pellet is on the upper side of the tube. Since viral pellets may be difficult to visualize, remember on what side of the tube a pellet will have formed. Take a micropipette and remove last bit of medium while taking care not to agitate the opaque brown pellet that is visible on the side of the bottom of the ultracentrifuge tube.
14. Resuspend this pellet in 500 μ l of organoid culture medium supplemented with 10 mM nicotinamide, 10 μ M Chir99021, 10 μ M Y27632 and 8 μ g/ml polybrene. Resuspension in this medium is important since high titer virus is used to transduce organoids directly.
15. Optionally: freeze the virus in this medium aliquoted in two batches of 250 μ l in -80 °C.

3. Lentiviral Transduction of Organoids

Day 0:

1. Split a full 0.95 cm² well of organoids two days prior to transduction into a new well, aiming to obtain approximately 50 small organoids. Split organoids according to previously published protocol¹ (Figure 3A, B).
2. Supplement organoid culture medium with 10 μ M Chir99021 and 10 mM nicotinamide to obtain cystic hyper proliferative crypts (Figure 3C).
NOTE: Cystic crypts will develop best when freshly split organoids are grown in the presence of Chir99021.

Day 2:

3. Harvest organoids by pipetting up and down the matrigel and medium, thereby disrupting the mixture with a p1000 micropipette. Place the mixture in a 15 ml tube.
4. Disrupt further using Pasteur pipet in which the distal opening has been decreased by melting. Centrifuge organoids to pellet for 5 min at 100 x g.
NOTE: Dependent on the size of the centrifuge, use a different centrifuge speed. It is necessary to find the exact speed in which disrupted organoids are separated from the mixture.
5. Remove the supernatant and add 500 μ l of pre-warmed 1x trypsin (similar to 0.25% trypsin). Resuspend organoids in trypsin and incubate 3 min in a 37 °C water bath.
6. Inactivate trypsin by adding 3.5 ml of cell line culture medium (DMEM supplemented with 10% FCS, 1% penicillin/streptomycin and glutamine). Centrifuge for 5 min at 500 x g.

7. Remove the supernatant to leave pellet in approximately 20 μ l of medium. Transfer the organoids in this last small amount of medium into a well on a 48-wells plate.
8. Add high titer lentivirus in transduction medium as described in step 2.14, resuspend and continue to step 3.10. When encountering low transduction efficacy, step 3.9 may be added.
NOTE: For efficient transduction, typically add one aliquot of 250 μ l of high titer lentivirus to one well of organoids. This represents 50% of all harvested lentivirus from a single production as described in step 2 of this protocol.
9. Optionally: to enhance transduction efficacy, perform spinoculation by putting the 48 well plate containing organoids in a pre-warmed centrifuge on 32 °C and rotate at 600 x g for 1 hr.
10. Put organoid-virus mixture in culture incubator and incubate for 1 hr at 37 °C in a culture incubator to allow transduction.
11. Optionally: to further enhance transduction efficacy, incubate organoids for additional 3 hr at 37 °C in a culture incubator.
12. Add 1 ml of organoid culture medium and resuspend the organoid-virus mixture, transfer into a microcentrifuge tube and centrifuge in a microcentrifuge for 5 min at 850 x g to pellet organoids.
13. Remove the supernatant and resuspend the pellet in 20 μ l of ice-cold matrigel. Since the material solidifies when it becomes warmer, use pipette tips that are chilled by pipetting up and down ice cold PBS for a number of times.
14. Put the droplet in the middle of a well in a 48 wells plate and incubate in culture incubator at 37 °C for 15 min to solidify.
15. After 15 min, carefully add 250 μ l of organoid culture medium supplemented with 10 mM nicotinamide, 10 μ M Chir99021 and 10 μ M Y27632. Small disrupted organoid fragments will form into small cystic organoids within 24 hr.

Day 5:

16. Refresh medium and supplement with a selection antibiotic (for puromycin, use 4 μ g/ml).

Day 7:

17. Replace medium for standard organoid culture medium, supplemented with selection antibiotic.
NOTE: Budding will be complete 2-3 weeks after Chir99021 withdrawal. After selection, organoids may be grown without selection antibiotic.

4. Organoid RNA Preparation for Quantitative RT-PCR or Microarray

1. For RNA preparation, use a commercial kit according to manufacturer's protocol. Remove the medium from organoids and add 350 μ l of buffer RLT, supplemented with β -mercaptoethanol straight on the dome of matrigel containing organoids. Resuspend the material in RLT by pipetting using p1000 micropipette.
2. Perform further RNA prep according to manufacturer's protocol.
3. Optionally: increase RNA yield by amplification using Ovation Pico WTA system, requiring initial input of 50 μ g total RNA according to manufacturer's protocol.
4. Optionally: for quality control prior to RNA microarray, run samples on a 2100 bioanalyzer using an eukaryote total RNA nano chip, aiming for an RNA integrity number (RIN) of 8.5 or more (**Figure 5**) according to manufacturer's protocol.

5. Processing Organoids for Paraffin Embedding and Immunohistochemistry

1. Prior to initiation of organoid embedding pre-warm an aluminum block with holes fitting 12 mm diameter glass tubes in incubator at 70 °C to keep paraffin liquid.
2. Take a single well with full-grown organoids and remove the medium, leaving the embedded organoids intact.
3. Add 1 ml of 4% paraformaldehyde in PBS straight to well and fix at 4 °C anywhere from 1 hr to overnight.
4. Replace paraformaldehyde with 1 ml of ice cold PBS.
Optionally: keep fixed organoids in PBS up to 1 week at 4 °C after this step.
5. Resuspend fixed organoids in 1 ml of PBS and place into glass vial.
6. Let organoids sink to bottom for 1 min, decant PBS and replace with 70% ethanol in which a couple of droplets of eosin solution are dissolved to enable visualization of organoids throughout the embedding process.
7. Leave organoids in 70% ethanol at room temperature. After 30 min, remove 70% ethanol by carefully decanting, being able to visualize the organoids by eye because of slight pinkish eosin color. Replace embedding solution with 96% ethanol.
8. Repeat step 5.7, each time replacing the embedding solution with the next one. Pass organoids through the following solutions subsequently: 70% ethanol, 90% ethanol, 96% ethanol, 100% ethanol, 100% ethanol, xylene, xylene.
9. Decant the last xylene wash and pour paraffin into the tube. Put the tube immediately in the pre warmed aluminum block at 70 °C for 30 min and replace paraffin with new clean paraffin.
10. Pour paraffin off and pipet organoids into the paraffin block mold using a pre-warmed Pasteur pipet with large opening. Keep Pasteur pipet warm using a Bunsen burner. Place all organoids in the paraffin block mold in a small layer of liquid paraffin.
11. As much as possible, try to manipulate all organoids towards the center of the paraffin block mold using a warmed dissection needle. Keep dissection needle warm using Bunsen burner.
12. When localization of organoids in the mold is satisfactory, chill mold slightly to solidify the paraffin layer.
13. Finish the block by pouring more paraffin on top and add a standard histological embedding cassette.

Representative Results

Organoid lentiviral transduction

The technique of organoid transduction using lentiviral particles depends on correct handling of organoids prior and during transduction. Organoids (**Figure 3A**) were cultured and they were disrupted into single crypts (**Figure 3B**). As previously reported, these single crypts,

when cultured in the presence of the GSK3 inhibitor Chir99021 became cystic crypts⁹ (**Figure 3C**). Subsequently organoids were trypsinized to allow penetration of virus particles to single cells. When transducing cells with lentiviral particles, a number of methods may be tried to enhance transduction efficacy such as spinoculation or prolonged incubation. High titer PGK-eGFP lentivirus was used to enable visualization of transduction efficacy by fluorescent microscopy. Transduction efficacy of organoids with this plasmid was high and approached 100% (**Figure 4E, F**). Improving efficacy using spinoculation (**Figure 4A, B**) or prolonged incubation with lentiviral particles (**Figure 4C, D**) did therefore not yield additional value.

RNA extraction

Next organoids for RNA extraction were grown. Full-grown organoids were harvested that were subjected to gamma irradiation or control treatment to show reduced RNA integrity (**Figure 5**). Upon irradiation with 6 Gy, RNA is degraded and compared to control treated organoids, the RNA integrity number (RIN) is reduced.

Immunohistochemistry on paraffin embedded organoids

After incubating organoids for 2 hours in culture medium supplemented with BrdU, organoids in formalin were fixed and processed for immunohistochemistry (**Figure 6**). Using mouse anti BrdU, proliferative cells could be observed in crypt-segments of organoids and not in the differentiated compartment.

Figure 1: Schematic of lentivirus production for organoid transduction. As written in protocol part 2, in this schematic, the most critical steps of virus production are represented with protocol step numbers and timing.

Figure 2: Schematic of lentiviral transduction of organoids. As written in protocol part 3, in this schematic, the most critical steps of organoid transduction are represented with protocol step numbers and timing.

Figure 3: Organoids prior to and during transduction. Normally growing organoids (A) are split into densely growing small organoids (B) that become cystic after incubation with Chir99021 for a number of days (C). Upon dissociation of these organoids using trypsin, single cells and small clumps of cells remain (D) that are transduced subsequently. Scale bar 100 μ m.

Figure 5: Representative result of RNA preps from organoids on bioanalyzer. Note strong demarcation of bands representing high RNA integrity in lanes 2-4 and smear around bands on lanes 5-7 representing log RNA integrity. RNA integrity number (RIN) of bands 2-4 is 8.7, 9.2 and 9 respectively, whereas the RIN of bands 5-7 is 6.4, 6.6 and 5.5.

Figure 6: Immunohistochemical analysis of formalin fixed paraffin embedded organoids. Formalin fixed paraffin embedded 4 μ m section of organoids cultured in the presence of BrdU for two hours and fixed subsequently. Section is stained with anti-BrdU antibody. Scale bar 100 μ m. [Please click here to view a larger version of this figure.](#)

	Basic medium	Additions
Cell line culture medium	DMEM	10% FCS
		1% penicillin/streptomycin
		2 mM Glutamine
Organoid culture medium	Advanced DMEM F12	HEPES
		1% penicillin/streptomycin
		1x glutamax
		1% N2 supplement
		2% B27 supplement
		125 nM n-acetyl cysteine
		mouse Egf (50 ng/ml)
		10% Nog-Fc conditioned medium (equivalent to 100 ng/ml)
		10% Rspo1-Fc conditioned medium (equivalent to 500 ng/ml)

Table 1: Culture medium composition.

Discussion

The current video protocol describes lentiviral transduction of organoids from primary intestinal epithelium and downstream analysis of these organoids using quantitative RNA techniques and immunohistochemistry.

Lentiviral transduction is often performed in adherent or floating cells in culture plates. Since the three-dimensional structure of organoids renders them difficult to penetrate by viral particles, a number of methods to increase efficacy are used. Pretreatment of organoids using Chir99021 increases proliferation and thereby viability after transduction. It is important to maintain small cell clusters after trypsinization, since single cells have decreased survival. In contrast to viral transductions using murine retrovirus, lentiviral transductions are more efficient and ordinarily do not require spinoculation, a technique known to increase transduction efficacy in ES cells¹⁰. When using murine retroviruses or when lentiviral transduction yields low efficacy, spinoculation can optionally be utilized to increase transduction rate. We do not regularly assess virus titer, since all available virus from a single round of production for transduction of organoids derived from maximally two 0.95 cm² wells was used. In addition, generally use antibiotic selection for removal of non-transduced cells. Assessment of viral titer may however be of value when encountering problems with transduction efficacy.

Identical to adherent cells, the level of selection antibiotic can increase the number of viral integrations and thereby expression of the transgene. Use high-level puromycin (10 µg/ml) to obtain high transgenic expression and effective knockdown, but when organoids exhibit signs of toxicity, lower levels may be used (minimally 1 µg/ml). In addition, alternative selection antibiotics may be used or selection may be omitted, although transduction is likely to be incomplete and this may not result in long-term stable expression. Furthermore, since lentiviral integration in the genome is heterogeneous throughout a population of cells, expression of the transgene may be subdued to changes after culturing cells for a prolonged time. This may result from selective pressure by expression of the transgene. Although antibiotic selection limits these changes, they may remain, especially in the case of constitutive expression of transgenes. This disadvantage may be overcome by single cell cloning of organoids, but this technique is time consuming. Lentiviral transduction can be performed with a wide variety of plasmids that result in either stable or inducible expression of transgenes. These transgenes are usually expressed from ubiquitous promoters, such as the CMV or the PGK promoter and may result in supranatural expression. Similar to transgenic overexpression in cancer cell lines or model animals, caution is warranted interpreting results from overexpression.

For experiments that require RNA, the low number of viable cells normally grown in a single 0.95 cm² well (standard surface area of 48 well) in contrast to adherent cells that can easily be grown in large flasks is the rate-limiting factor for quality and multitude of downstream analysis. We find that normally, a single well of approximately 50 full grown organoids yields between 0.4 µg and 1 µg total RNA, being sufficient for quantitative RT-PCR and RNA microarray without further amplification. Certain treatment regimens or genetic alterations can reduce the RNA content significantly however. A first step to increase total RNA is pooling of multiple wells.

For paraffin embedding of organoids, it is critical to obtain sufficient material in order to visualize organoids during the process. Addition of minute amounts of eosin to fixed organoids allows visualization of organoids during the whole process, but is not required for normal embedding and may be omitted when this step interferes with further analysis. Usually, paraffin-embedding cassettes have holes in them to allow flow of fixative and process solutions to the tissue in the cassette.

Lentiviral organoid transduction and preparation for downstream standard techniques increases scientific potential of these cultures and raises the standard in cell culture to three-dimensional techniques from primary intestinal epithelium. The range of downstream experiments to be performed is however not limited to techniques described in the current protocol and may encompass all techniques performed on cell lines or mice, given the amount of cellular material generated by culture is sufficient. In research on specific genetic elements and their function in the intestinal epithelium, homologous recombination techniques in model animals, most notably mice, remain the gold standard. Cultures of organoids do not contain mesenchymal or immunological niche cells and cultured crypts are ever expanding, contrasting the situation found *in vivo*. Nonetheless, these cultures have raised the quality of *in vitro* findings greatly and taking into account the myriad downstream techniques that can be performed, organoid culture has and will greatly enhance research on the intestinal epithelium.

Disclosures

The authors have nothing to disclose.

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