Derivation of snake venom gland organoids for in vitro venom production

Puschhof, Jens; Post, Yorick; Beumer, Joep; Kerkkamp, Harald M.; Bittenbinder, Matyas; Vonk, Freek J.; Casewell, Nicholas R.; Richardson, Michael K.; Clevers, Hans

published in
Nature Protocols
2021

DOI (link to publisher)
10.1038/s41596-020-00463-4

document version
Publisher's PDF, also known as Version of record

nature protocols

PROTOCOL

https://doi.org/10.1038/s41596-020-00463-4

Check for updates

Derivation of snake venom gland organoids for in vitro venom production

Jens Puschhof[1,2,7], Yorick Post[1,2,7], Joep Beumer[1,2,7], Harald M. Kerkkamp[3,4], Matyas Bittenbinder[3,5], Freek J. Vonk[3], Nicholas R. Casewell[6], Michael K. Richardson[4] and Hans Clevers[1,2]✉

More than 400,000 people each year suffer adverse effects following bites from venomous snakes. However, snake venom is also a rich source of bioactive molecules with known or potential therapeutic applications. Manually 'milking' snakes is the most common method to obtain venom. Safer alternative methods to produce venom would facilitate the production of both antivenom and novel therapeutics. This protocol describes the generation, maintenance and selected applications of snake venom gland organoids. Snake venom gland organoids are 3D culture models that can be derived within days from embryonic or adult venom gland tissues from several snake species and can be maintained long-term (we have cultured some organoids for more than 2 years). We have successfully used the protocol with glands from late-stage embryos and recently deceased adult snakes. The cellular heterogeneity of the venom gland is maintained in the organoids, and cell type composition can be controlled through changes in media composition. We describe in detail how to derive and grow the organoids, how to dissociate them into single cells, and how to cryopreserve and differentiate them into toxin-producing organoids. We also provide guidance on useful downstream assays, specifically quantitative real-time PCR, bulk and single-cell RNA sequencing, immunofluorescence, immunohistochemistry, fluorescence *in situ* hybridization, scanning and transmission electron microscopy and genetic engineering. This stepwise protocol can be performed in any laboratory with tissue culture equipment and enables studies of venom production, differentiation and cellular heterogeneity.

Introduction

Since their development ten years ago, adult stem cell–derived organoids have been established from most epithelial tissues of humans and mice[1,2]. Their ability to maintain cellular heterogeneity, self-organize into three-dimensional structures and be maintained long-term have made organoids invaluable tools in biomedical research[3].

The snake venom gland is an epithelial organ with a clear function: the production of toxins. Snake venom has a devastating impact on more than 400,000 snakebite victims each year, killing more than 100,000 and causing lasting disabilities in many survivors[4]. On the other hand, it is also a rich resource for the identification of bioactive molecules for drug development. For these reasons, understanding venom production and function, including through the derivation of tissue models of the snake venom gland, is an active area of research[5–7]. While previous in vitro models of the snake venom gland have shown the ability to derive cell lines and suspension cultures, the broad utility of these models has been hampered by their short lifespan or by a lack of representation of tissue complexity[5–7].

This protocol aims to address this need by providing detailed instructions for the derivation of organoids from snake venom glands, their maintenance and how to extract the venom produced by the organoids. We first used this protocol to unravel the cellular heterogeneity of the venom gland of the Cape coral snake *Aspidelaps lubricus* and several other species belonging to both the elapid and viperid families[8]. The faithful representation of cell types and long-term preservation of regional features from proximal and distal parts of the gland that we demonstrated in our previous work underline the validity of snake venom gland organoids (VGOs) as an in vitro model of the venom

[1]Hubrecht Institute, Royal Netherlands Academy of Arts and Sciences (KNAW) and UMC Utrecht, Utrecht, the Netherlands. [2]Oncode Institute, Hubrecht Institute, Utrecht, the Netherlands. [3]Naturalis Biodiversity Center, Leiden, the Netherlands. [4]Institute of Biology Leiden, Department of Animal Science and Health, Leiden, the Netherlands. [5]Amsterdam Institute for Molecules Medicines and Systems, Division of BioAnalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Faculty of Sciences, Vrije Universiteit Amsterdam, Amsterdam, the Netherlands. [6]Centre for Snakebite Research & Interventions, Department of Tropical Disease Biology, Liverpool School of Tropical Medicine, Liverpool, UK. [7]Co-first author: Jens Puschhof, Yorick Post, Joep Beumer. ✉e-mail: h.clevers@hubrecht.eu

Box 1 | Comparison with alternative non-mammalian stem cell systems

Induced-pluripotent stem cells (iPSCs) can be generated from chicken fibroblasts by overexpression of the 'Yamanaka'-factors—a set of transcription factors first described and commonly used in mammalian protocols—plus manipulation of additional genes[20]. A similar strategy was recently used to generate iPSCs from fish fibroblasts[21].

The only other publication describing ASC-derived organoids has been the demonstration that chicken intestinal organoids can grow in virtually identical growth factors to those used to culture mammalian intestinal tissue[22].

gland. This protocol can be performed in any laboratory equipped for cell culture and by anyone with basic training in organoid culture techniques.

Development of the protocol

The protocols for establishing, maintaining and differentiating VGOs are inspired by and highly similar to those previously published for human organoid systems[9,10]. While similar to human pancreatic organoid medium[10], the snake VGO medium comprises a unique mixture of mammalian growth factors which have been optimized for the expansion and differentiation of VGOs[8]. The enzyme digestion steps of the dissociating procedures have been optimized to allow all protocol steps to be performed at room temperature or 32 °C, as higher temperatures significantly decrease venom gland cell viability.

Comparison with alternative snake-derived methods

There are several alternative methods by which venom can be obtained or produced. For some applications these alternative methods are more suitable than our protocol, particularly if cost is a consideration, or if rapid large-scale production of a specific venom is required.

Crude venom can be obtained by manually 'milking' snakes. Currently, milking snakes yields the largest amounts of any protocol, and guarantees broad coverage of the mixture of toxins present in the venom. However, we anticipate that producing venom from organoids would be preferable for applications that require eventual scalability beyond the yields possible with milking methods or if the precise composition of the product is important, as our protocol facilitates the modification of individual toxins.

Existing snake venom gland cell lines permit modeling of some characteristics of the original organ. These cultures, grown in suspension as single cells[5] or follicular structures[6,7], can be grown for months to years at lower cost than organoids and produce some venom. An advantage of organoids is that in contrast to cell line cultures, adult stem cells persist in the culture, enabling both long-term culture and genetic modification.

The culture conditions for organoids are fully defined, which can be advantageous for some applications. The composition of organoid-derived venom can be modified by the growth medium used[8], facilitating more targeted differentiation of cell types. This feature enables more detailed characterization of specific cell types and greater scalability of venom production; however, it also represents a potential limitation in that conditions need to be optimized to generate specific venom compositions. Nevertheless, the organoid culture approach has been successfully applied to ten different species, demonstrating its robustness and more general applicability[8]. Genetic modification, targeted differentiation of specific cell types, and manipulation of the production of specific venoms have not been possible to date using cell line models or explants of the venom gland.

However, one disadvantage of snake venom gland organoids is that that they only contain epithelial cell types, thus prohibiting the modeling of interactions with surrounding muscle and nerve cells. The organoids maintain regional characteristics[8], so the starting material should be carefully considered to ensure the appropriate part of the venom gland is modeled. This characteristic can also be advantageous, though: for example, we applied this system to study the cellular and regional heterogeneity of the venom gland[8].

As snake VGO cultures have only recently been developed, all applications for large-scale venom production are speculative. We envision the scalable production of venom in a chemically defined medium for the tightly controlled generation of antivenom. Furthermore, the possibility to genetically engineer venom gland organoids might pave the way to the production of optimized toxins.

For completeness we also include a summary of the limited number of publications to date that describe other non-mammalian stem cell systems (see Box 1).

Table 1 | Overview of species, starting material and time in culture achieved to date for snake venom gland organoids

Species	Adult/embryo	Duration of successful culture
Aspidelaps lubricus cowlesi	Embryo	24 months
Naja nivea	Adult	6 months
Bitis arietans	Adult	3 months
Naja atra	Embryo	3 months
Crotalus atrox	Adult	1 month
Deinagkistrodon acutus	Adult	1 month
Echis ocellatus	Adult	1 month
Naja annulifera	Adult	1 month
Naja pallida	Adult	1 month
Naja haje	Embryo	1 week

In summary, we anticipate that snake venom gland organoids will serve as a useful platform to elucidate the cell biology of the venom gland and to produce and modify venom under controlled conditions for therapeutic purposes.

Experimental design

Snake venom gland organoids can be established from a new biopsy of an embryonic or adult snake: in these situations, begin the procedure at Step 1A or B, respectively. If the starting material is received in cryopreserved form, the procedure should be started at Step 23B(ix). The euthanization of adult snakes was described in detail previously[7] and is not included here. In our experience, suitable biopsies can be obtained from snakes that died of natural causes within a day of their death. Nevertheless, when using adult snake-derived biopsies, storage and transport times should be minimized. Extensive washing might be necessary if cytotoxins are abundant in the venom, to avoid decreased viability of the initial organoid culture.

Late-stage embryonic biopsies, harvested after retrieving an embryo from an egg, are at less risk of bacterial infections of the culture in the early stages of establishing a new organoid line than are cultures derived from biopsies from adult snakes. We have previously observed the toxin expression profiles in late-stage embryonic biopsy cultures to be similar to those seen in adult snakes[8], with a negligible effect of cytotoxins on cell viability. We therefore recommend late-stage embroyos as the starting material for venom gland organoids.

Although we have not yet attempted this, we speculate that deriving single viable adult stem cells from snake venom might be less invasive than taking a biopsy and hence might be an even more versatile starting point from which to derive organoids. Similar concepts have proven practical for kidney organoids derived from single cells in urine[11].

The procedure we describe here successfully supported the outgrowth of venom gland organoids from all snake species tested so far (Table 1)[8]. We speculate that it might also support the growth of organoids from other reptile species and potentially even more evolutionarily distant animals.

In our experience, organoids grow at a wide range of speeds. For our studies to date we were only able to obtain one or two tissue samples from each species, and the snake tissue received was from snakes of various ages and sex. In addition, the time required to isolate follicular structures (Step 6) varied and different batches of media were used. It is thus hard to define the exact contribution of individual factors to the rate of growth. Users should thus expect organoids from different source materials to grow at different speeds. We recommend moving to subsequent stages of the procedure when organoids reach the indicated size (see relevant steps in the procedure, typically 50–200 μm) rather than at a particular timepoint.

Organoid morphology, dilution factor during passaging and toxin gene expression best determine whether organoids are successfully established. In the expansion medium used for establishment and propagation, organoids should grow as spheres. Denser and less organized structures can be signs of contamination with non-epithelial cell types or suboptimal media conditions. Once a snake VGO line has reached the stable growth phase (usually 2 passages after establishment), it should be split by a factor of ~1:3 every 7–14 d. Significantly lower expansion speed can indicate suboptimal media

conditions. Finally, the identity of the cultured organoids should be established as early as possible using quantitative real-time PCR (qPCR) for cell type markers. Toxin genes are well suited to distinguishing venom gland organoids from any other tissue. As reference, RNA extracted from primary venom gland and non-venom gland tissue should be assessed. Any expression significantly over the non-venom gland tissue level is indicative of the presence of venom gland organoids.

Many of the proposed applications presented in this protocol require VGO cells to be differentiated towards venom-producing cell types. Withdrawal of stem cell-promoting growth factors is sufficient to induce differentiation of venom-producing cells within 7 d. Quantitative real-time PCR for toxin genes can be used to track differentiation dynamics and determine the ideal timepoint for differentiation towards production of specific toxins. Upon differentiation, organoids stop proliferating and cannot be passaged further upon reaching terminal differentiation. Differentiated organoids can be maintained in a venom-producing state for up to 3 weeks until excessive cell death becomes apparent. An overview of the workflow connecting the steps of this protocol is shown in Fig. 1.

Materials

Biological starting material

Dead adult or embryonic snake. Detailed guidelines for the euthanasia of snakes has been published previously[8]. *Caution*: Handling venomous snakes is extremely dangerous and should only be performed by trained experts. Approval from the relevant animal experiment board must be obtained. It is also important that all relevant guidelines on animal experiments are complied with and that each species is handled appropriately (CITES (https://www.cites.org/eng) and Nagoya protocol (https://www.cbd.int/abs/) regulations may apply). Ensure that the snake is humanely euthanized according to the recommendations of the relevant ethics authority. We obtained approval for our previous studies[8] from the following institutions: *Crotalus atrox* from the Natural Toxins Research Center in Texas, USA approved by Institutional Animal Care and Use Committee (IACUC); *Echis ocellatus* and *Deinagkistrodon acutus* from the Liverpool School of Tropical Medicine, UK approved by the UK Home Office and the LSTM Animal Welfare and Ethical Review Board; *Naja pallida, N. nivea* and *Bitis arietans* were maintained in captivity at SERPO, Rijswijk, NL in compliance with local animal welfare guidelines. Residual post-mortem material from venom glands was used to establish organoids; *N. atra, N. annulifera* and *A. lubricus cowlesi* embryonic material was obtained from local breeders in The Netherlands. Local animal research law (Wet op Dierproeven, WOD, artikel 1b lid 5a) enabled the use of embryonic reptile material for research purposes.

Reagents

Chemicals, peptides, recombinant proteins, antibodies and kits

- Advanced Dulbecco's Modified Eagle Medium (DMEM)/F12 (Thermo Scientific, cat. no.12634010)
- B-27 Supplement (Thermo Scientific, cat. no. 17504044)
- GlutaMAX (Thermo Scientific, cat. no. 35050061)
- HEPES (Thermo Scientific, cat. no. 15630080)
- Penicillin-streptomycin (Thermo Scientific, cat. no. 15140122)
- Primocin (Invivogen, cat. no. ant-pm-2)
- *N*-acetyl-L-cysteine (Sigma-Aldrich, cat. no. A9165)
- Nicotinamide (Sigma-Aldrich, cat. no. N0636)
- Noggin-conditioned medium (U-Protein Express, cat. no. N002)
- R-spondin conditioned medium (U-Protein Express, cat. no. R001)
- Human epidermal growth factor (EGF) (Peprotech, cat. no. AF-100-15)
- A83-01 (Tocris, cat. no. 2939)
- Prostaglandin E2 (PGE2) (Tocris, cat. no. 2296)
- Gastrin I (Tocris, cat. no. 3006)
- Human fibroblast growth factor-10 (FGF-10) (Peprotech, cat. no. 100-26)
- Forskolin (Tocris, cat. no. 1099)
- Y-27632 dihydrochloride (Abmole, cat. no. M1817)
- Gentamicin (Sigma-Aldrich, cat. no. G1397)
- Ciprofloxacin (Sigma-Aldrich, cat. no. 17850)
- Erythromycin (Sigma-Aldrich, cat. no. E5389)

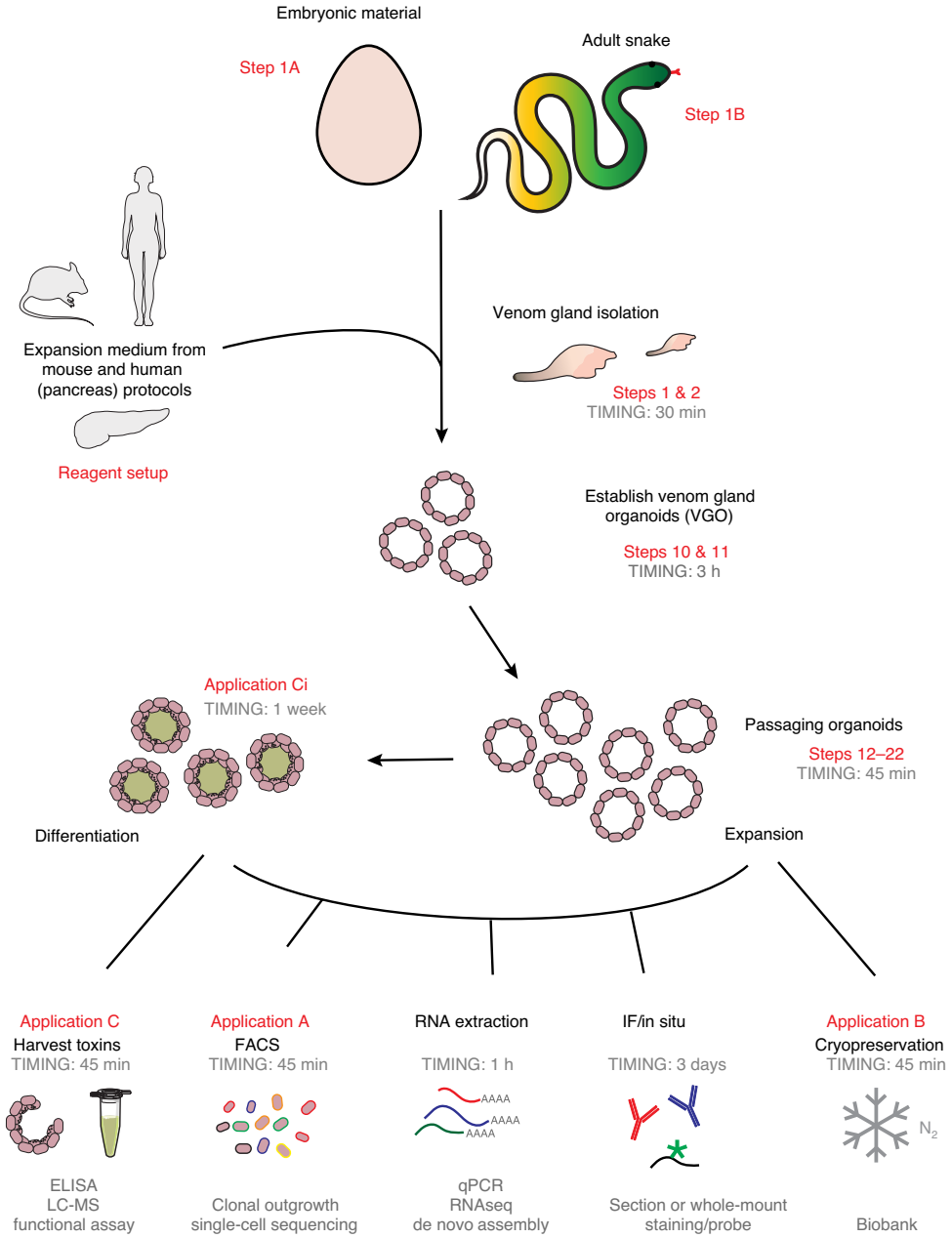

Fig. 1 | Overview of working with snake venom gland organoids. Top: schematic representation of the steps involved in establishing organoids from primary venom gland tissue and passaging and expanding organoids. Middle: differentiation of organoids for higher venom production. Bottom: selected research applications for organoids in expansion medium and/or differentiation medium.

- Azithromycin dehydrate (Sigma-Aldrich, cat. no. PZ0007)
- FBS (Thermo Scientific, cat. no. 16140071)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, cat. no. D2650)
- DMEM (Thermo Scientific, cat. no. 11965092)
- Collagenase from *Clostridium histolyticum* (Sigma-Aldrich, cat. no. C9407)
- Basement membrane extract (BME), growth factor reduced, type 2 (R&D Systems, cat. no. 3533-001-02)
- DAPI (Thermo Scientific, cat. no. D1306; RRID: AB_2629482; dilution 1:1,000)
- Rabbit anti-SOX2 (Millipore, cat. no. AB5603; RRID: AB_2286686; dilution 1:100)
- Rabbit anti-β-tubulin (Santa Cruz, cat. no. sc-9104; RRID: AB_2241191)

- Phalloidin-Alexa 647 (Thermo Scientific, cat. no. A22287; RRID: AB_2620155; dilution 1:1,000)
- TRIzol (Thermo Scientific, cat. no. 15596026) **! CAUTION** Hazardous upon inhalation, ingestion or skin contact. Wear protective gear and open in fume hood.
- RNeasy Mini Kit (QIAGEN, cat. no. 74104)

Equipment

- Falcon tubes, 15 mL
- Falcon tubes, 50 mL
- Polystyrene round-bottom tube with cell-strainer caps, 5 mL (Falcon)
- Microcentrifuge tubes, 1.5 mL
- Plates, 6-well (Greiner Bio-One, cat. no. 657 160)
- Plates, 12-well (Greiner Bio-One, cat. no. 665 180)
- Plates, 24-well (Greiner Bio-One, cat. no. 662 160)
- Plates, 48-well (Greiner Bio-One, cat. no. 677 180)
- Cell culture dishes, 100 × 20 mm (Greiner Bio-One, cat. no. 664 160)
- Glass pasteur pipettes (VWR, cat. no. 612-1701)
- EVOS Cell Imaging System (Thermo Fisher, M5000)
- Dissection microscope (Leica, MZ75)
- Dissection tools (NeoLab)
- Disposable scalpels (Swann-Morton, 0501)
- Centrifuge (Eppendorf, 5810R)
- Centrifuge (Eppendorf, 5424)
- 5% CO_2 incubator (at 32 °C)
- 32 °C shaking platform
- Biosafety cabinet

Reagent setup

Snake venom gland organoid expansion medium

Expansion culture medium is used to establish and maintain of VGOs. To make expansion medium, supplement AdDMEM/F12 with B-27 Supplement (1×), GlutaMAX (1×), HEPES (1×), 100 U/mL penicillin-streptomycin, 100 µg/mL primocin, 1.25 mM N-acetylcysteine, and 10 mM nicotinamide. The following growth factors should be added: 2% (vol/vol) Noggin conditioned medium, 2% (vol/vol) Rspo3 conditioned medium, 50 ng/mL EGF, 0.5 µM A83-01, 1 µM PGE2, 100 nM gastrin, 100 ng/mL FGF10. Depending on species, adding 1 µM Forskolin (FSK) can have a beneficial effect (we have found this to be beneficial for *N. nivea*). ▲ **CRITICAL** After every step in which the organoids are dissociated to single cells, 10 µM Y-27632 should be added for at least 5 d or until cystic organoids have formed again, to prevent anoikis. ▲ **CRITICAL** The organoid medium should be stored at 4 °C and used within 1 month of preparation. It can be shipped in frozen or liquid form and used within the same timeframe after thawing. Avoid freeze–thaw cycles.

Antibiotics cocktail

During establishment of an organoid culture, and if bacterial contamination occurs at any stage, the following antibiotics should be added to snake venom gland expansion medium: 50 µg/mL gentamicin, 2.5 µg/mL ciprofloxacin, 20 µM erythromycin, and 100 nM azithromycin. ▲ **CRITICAL** The organoid medium with added antibiotics should be stored at 4 °C and used within 1 month of preparation. It can be shipped in frozen or liquid form and used within the same timeframe after thawing. Avoid freeze–thaw cycles.

Snake venom gland organoid differentiation medium

Differentiation medium is used to increase the number and maturity of venom-producing cells in snake VGOs. As for expansion medium, to make differentiation medium, supplement AdDMEM/F12 with GlutaMAX (1×), HEPES (1×), 100 U/mL penicillin-streptomycin, 100 µg/mL Primocin, 1.25 mM N-acetylcysteine, 10 mM nicotinamide and 1 µM PGE2. ▲ **CRITICAL** The organoid medium should be stored at 4 °C and used within 1 month of preparation. It can be shipped in frozen or liquid form and used within the same timeframe after thawing. Avoid freeze–thaw cycles.

Organoid freezing medium
To cryopreserve snake VGOs, a 2× freezing medium stock is prepared. For 2× freezing medium, add 1 volume of DMSO to 4 volumes of FBS. Freezing stock medium can be stored at 4 °C for more than 6 months. ▲CRITICAL The organoid medium should be stored at 4 °C and used within 1 month of preparation. It can be shipped in frozen or liquid form and used within the same timeframe after thawing. Avoid freeze–thaw cycles.

Digestion blocking buffer
Add 1 part of FBS to 4 parts of DMEM and add Y-27632 to a concentration of 10 μM. ▲CRITICAL The blocking buffer should be stored at 4 °C and used within 3 months of preparation. After addition of Y-27632, the medium should be used within 1 month. It can be shipped in frozen or liquid form and used within the same timeframe after thawing. Avoid freeze–thaw cycles.

Preparation and storage of growth factor stocks

▲CRITICAL Consult manufacturers' instructions for maximum storage time for all of the following.

B-27 supplement
Provided as 50× stock solution. Can be stored at −20 °C for 1 year.

***N*-acetylcysteine**
Dissolve 81.5 mg per mL H_2O to prepare a 400×, 500 mM stock solution. Can be stored at −20 °C for 1 month.

Nicotinamide
Dissolve 1.2 g in 10 mL PBS to prepare a 100×, 1 M stock solution. Store at −20 °C, stable for 1 year.

Noggin-conditioned medium
Provided as a 50× stock solution. Store at −20 °C, stable for at least 2 months.

R-spondin-conditioned medium
Provided as a 50× stock solution. Store at −20 °C, stable for at least 2 months.

Human EGF
Dissolve 1 mg in 2 mL PBS + 0.1% (wt/vol) BSA to prepare a 10,000×, 0.5 mg/mL stock solution. Store at −20 °C, use within 2 months.

A83-01
Dissolve 2 mg in 950 μl DMSO to obtain a 50,000×, 5 mM stock solution. Store at −20 °C, recommended use within 1 month.

PGE2
Dissolve 10 mg in 2.84 mL DMSO to prepare a 10,000×, 10 mM stock solution. Store at −20 °C, recommended use within 1 month.

FGF-10
Dissolve 500 μg in 5 mL PBS + 0.1% (wt/vol) BSA to prepare a 10,000×, 0.1 mg/mL stock solution. Store at −20 °C, use within 2 months.

Y-27632
Dissolve 50 mg in 1.5 mL H_2O to prepare a 10,000×, 100 mM stock solution. Store at −20 °C, recommended use within 1 month.

Collagenase
Dissolve 20 mg Collagenase in 1 mL of adDMEM/F12 to make a 20 mg/mL stock solution. Store at −20 °C, use within 2 months.

Forskolin
Dissolve the powder in DMSO to prepare a 10,000×,10 mM stock solution. Store at −20 °C, recommended use within 1 month.

Gastrin I
Dissolve the powder in PBS + 0.1% BSA to prepare a 10,000×,100 μM stock solution. Store at −20 °C, use within 2 months.

Gentamicin
Provided as a 50 mg/mL 1,000× stock solution. Store at 4 °C.

Ciprofloxacin
Dissolve in DMSO to a 10,000× stock with a concentration of 25 mg/mL. Store at −20 °C.

Erythromycin
Dissolve in DMSO to a 10,000× stock with a concentration of 200 mM. Store at −20 °C.

Azithromycin
Dissolve in DMSO to a 100,000× stock with a concentration of 1 mM. Store at −20 °C.

Procedure

Establishing a snake venom gland organoid line from adult or embryonic tissue ● Timing 3 h (2 h hands-on)

! CAUTION Handling venomous snakes is extremely dangerous and should only be performed by trained experts. Approval from the relevant animal experiment board must be obtained, guidelines on animal experiments must be complied with and the species must be appropriately handled (CITES and Nagoya protocol regulations may apply).

! CAUTION Ensure the snake is humanely euthanized according to the recommendations of the relevant ethics authority. See the introduction for details of how we euthanized the snake species used to generate the data here and the data included in our previous publication[8].

▲ CRITICAL Detailed guidelines for the euthanasia of snakes and further description of how to dissect the venom gland is also provided in ref. [7].

1 Dissect venom glands from a freshly euthanized snake as described in option A for late-stage embryos or option B for adult snakes.
   (A) **For late-stage embryos**:
      (i) Clean all equipment and the outside of the eggs with 70% ethanol before the procedure to avoid contamination (Fig. 2a).
      (ii) Remove the late-stage embryo from the egg and cut the skin above the venom glands.
      (iii) Using scalpels, dissect the venom gland from the snake and transfer it to a 100 mm cell culture dish.
   (B) **For adult snakes:**
      (i) Clean all equipment and the outside of the sacrificed snake with 70% ethanol before the procedure to avoid contamination (Fig. 2a).
      (ii) Cut the skin above the venom glands. Using scalpels, dissect the venom gland from the snake and transfer it to a 100 mm cell culture dish.
      ■ PAUSE POINT Dissected glands can be kept for up to 1 d at 4 °C in DMEM containing the antibiotics cocktail and 10 μM of Y-27632, if required for transport. The time from dissection to organoid derivation should be minimized.
      ? TROUBLESHOOTING
2 Using scalpels, remove as much connective tissue, fat and other tissue parts as possible (Fig. 2b). If available, a stereomicroscope should be used to guide tissue identification during the dissection process. Some remaining stromal tissue is acceptable, as the organoid growth medium will only support the long-term growth of epithelial cells. Transfer the gland into a 10 cm cell culture dish and transfer dish to a sterile laminar flow cabinet.
3 If you wish to derive separate organoid lines from specific regions of the venom gland, cut perpendicularly to the proximal-distal axis to separate out regions and process separately from this stage on (Fig. 2c).
   ▲ CRITICAL Carry out this and subsequent steps inside a sterile laminar flow cabinet.
4 Further cut the venom gland into small pieces (ideally <1 mm) with scalpels.

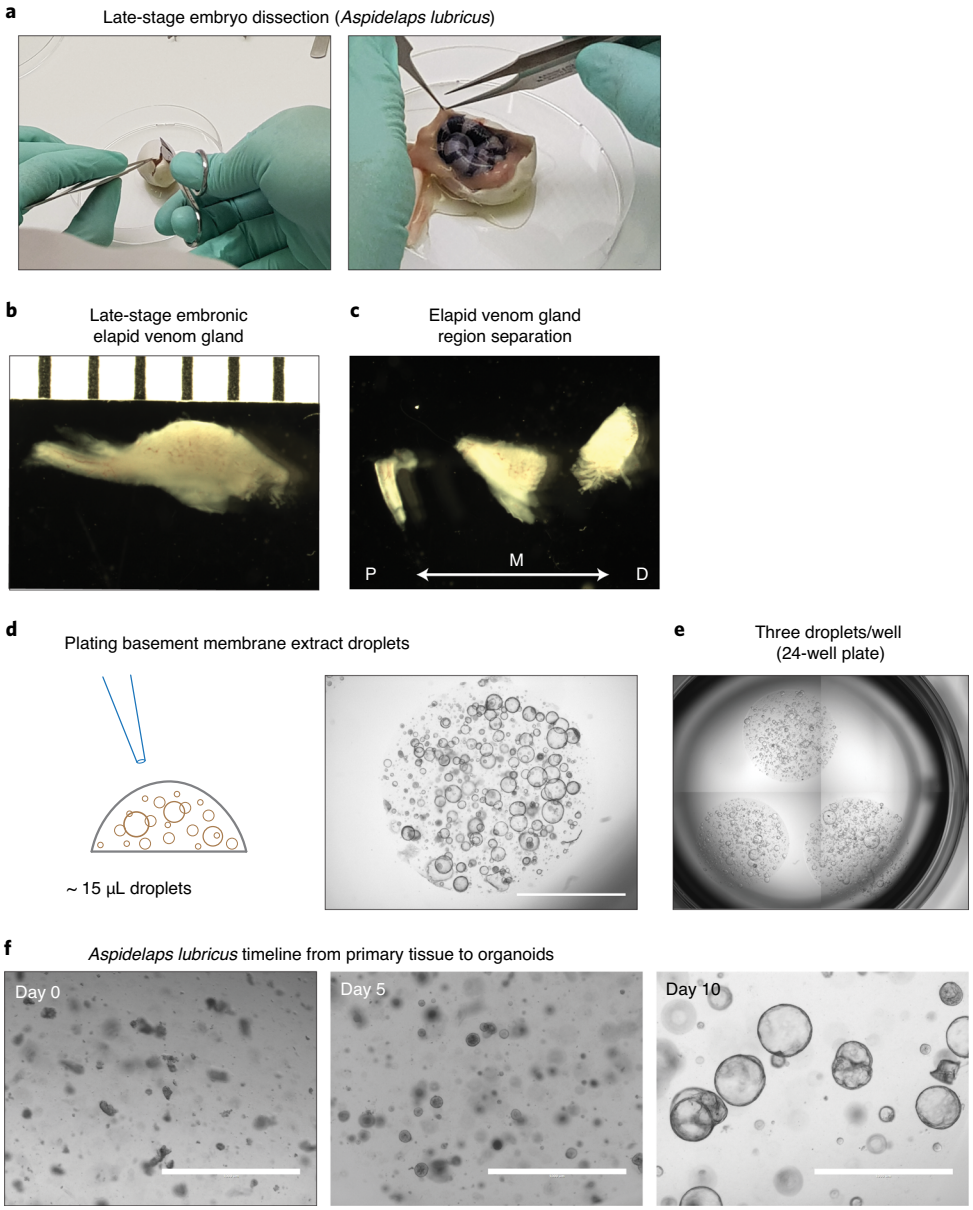

Fig. 2 | Establishment of snake venom gland organoids from primary tissue. a, Isolation and dissection of late-stage embryonic (~ 5 d before hatching) *A. lubricus* to obtain venom gland tissue under sterile conditions. **b**, Complete late-embryonic venom gland from *N. annulifera* after removal of muscle and connective tissue. Scale bar increments are 1 mm. **c**, *N. annulifera* venom gland (from b) after separation into proximal (P, ductal), middle (M) and distal (D) parts using microdissection. **d**, Plating of basement membrane extract (BME) as 3D environment for organoids to expand in. Left, illustration of side-view 15 uL droplet. Right, brightfield image of ~100 organoids in 15 µL droplet, view from above. Scale bar, 2 mm. **e**, View from above into a 24-well plate well with three BME droplets. **f**, Time course of organoid expansion after seeding of cells from *A. lubricus* venom gland cells in BME (passage 0) from day 0 to day 10. Scale bars, 1 mm.

5 Wash pieces three times with DMEM containing the antibiotics cocktail and 100 µg/mL Primocin.

6 Transfer tissue pieces to a 15 mL Falcon tube filled with 5 mL Collagenase in DMEM (1 mg/mL) containing 10 µM of Y-27632. Incubate for ~30 min at 32 °C under constant rocking (~120 r.p.m. on a tube rocker). Repeatedly harvest ~20 µL of solution and assess tissue digestion under a brightfield microscope (start checking after 15 min and repeat every 5–10 min). Stop the digestion once follicular structures become apparent and proceed to dissociation with the next step.

▲ **CRITICAL STEP** Avoid temperatures above 32 °C during the digestion process. While higher temperatures can enhance enzyme activity, if the cells produce a heat shock response their viability can be dramatically reduced (as shown in ref. [8]).

? TROUBLESHOOTING

7 Top up Falcon tube to 10 mL with DMEM containing the antibiotics cocktail and 100 µg/mL Primocin and centrifuge for 5 min at 4 °C and 300*g* to pellet tissue pieces. Remove supernatant by aspiration. Repeat this step twice to wash the fragments.
▲ **CRITICAL STEP** The digestion mixture can also be filtered using a 70 µm filter to remove larger undigested fragments. This is not required if the epithelium is dissected cleanly, but is highly advisable if tissue fragments do not dissociate properly.

8 Aiming for a density of ~150 organoid structures per 15 µL drop (shown in Fig. 2d), resuspend the fragments in ice-cold basement membrane extract (BME) (some DMEM can be left in the tube; the final BME concentration should be >60%). Plate in droplets of ~15 µL in a preheated sterile tissue culture plate. We recommend placing 1 droplet per well in a 48-well plate; 3 droplets per well in a 24-well plate (Fig. 2e); 7 droplets per well in a 12-well plate and 18 droplets per well in a 6-well plate.
▲ **CRITICAL STEP** Keep BME on ice and work quickly to avoid solidification prior to plating.

9 Carefully flip the plate bottom-up and place it in an incubator at 32 °C to ensure equal distribution of tissue fragments during solidification. Leave to solidify as 'domes' for 20–30 min.

10 Flip plate the right way up and add 500 µL of prewarmed expansion medium with 10 µM Y-27632 to each well of a 24-well plate (2 mL per well in a 6-well plate, 1 mL per well in a 12-well plate and 300 µL per well in a 48-well plate).

11 Refresh medium every 3 d, depending on organoid growth. Once cystic organoids with a diameter >200 µm are formed (usually within 2–10 d, Fig. 2f), move to the next step.
▲ **CRITICAL STEP** Ideally leave organoids to grow to >200 µm in average diameter after isolation before splitting. If the BME dome is cloudy with debris and stromal tissue, an earlier split can be advantageous.
? TROUBLESHOOTING

Passaging snake venom gland organoids ● Timing 45 min (25 min hands-on)

▲ **CRITICAL** When organoids are on average greater in size than >200 µm (typically 1–3 weeks after last split), they are ready to be passaged (Fig. 3a, I,II) as described in this section.
▲ **CRITICAL** As discussed further in the Anticipated Results section, in our experience, sufficient toxin transcripts are produced by VGOs being grown in expansion medium to confirm the identity of the organoids by qPCR. Thus, organoids can be further characterized by qPCR at this stage.

12 Remove culture medium and resuspend BME dome(s) in cold DMEM by pipetting with a P1000 pipette.

13 Transfer resuspended organoids into a 15 mL Falcon tube and top up to 10 mL with cold DMEM (Fig. 3a, II).

14 Centrifuge for 5 min at 4 °C at 300*g* to pellet organoids. The pellet should consist of two layers: a dense white pellet at the bottom which comprises the organoids and organoid fragments, as well as a transparent pellet of BME (Fig. 3a, III).
▲ **CRITICAL STEP** If the cell pellet is mixed with the BME phase after centrifugation, remove DMEM, resuspend in new ice-cold DMEM and centrifuge again until clear separation is reached. We have found mixing in ~10% of cases, but it can be almost completely avoided if the pellet is resuspended carefully in cold DMEM and the centrifugation is repeated.

15 Carefully aspirate DMEM and transparent layer of pellet.
▲ **CRITICAL STEP** The cell pellet might be attached to the lower part of the BME pellet. If the cell pellet is hard to separate from the BME pellet, we recommend using a P1000 pipette to carefully remove as much of the BME as possible.

16 Resuspend organoids in 1 mL of cold DMEM and disrupt them by repeated pipetting with a fire-polished glass pipette attached to a Pipetboy (Fig. 2a-IV); the opening of the glass pipette should be 0.5–1 mm after narrowing with a Bunsen burner. Alternatively, a P10 tip can be placed on top of a P1000 tip and used for mechanical disruption of the organoids. The organoid fragments in the mixture should get visibly smaller (more cloudy) after repeated pipetting. Assess by eye or with a small volume from the mixture under the microscope. Stop once no more individual particles are visible by eye.

17 Top up tube containing organoids to 10 mL with cold DMEM and centrifuge at 4 °C at 300*g* for 5 min to pellet organoid fragments (Fig. 3a, V).

18 Aspirate DMEM supernatant.

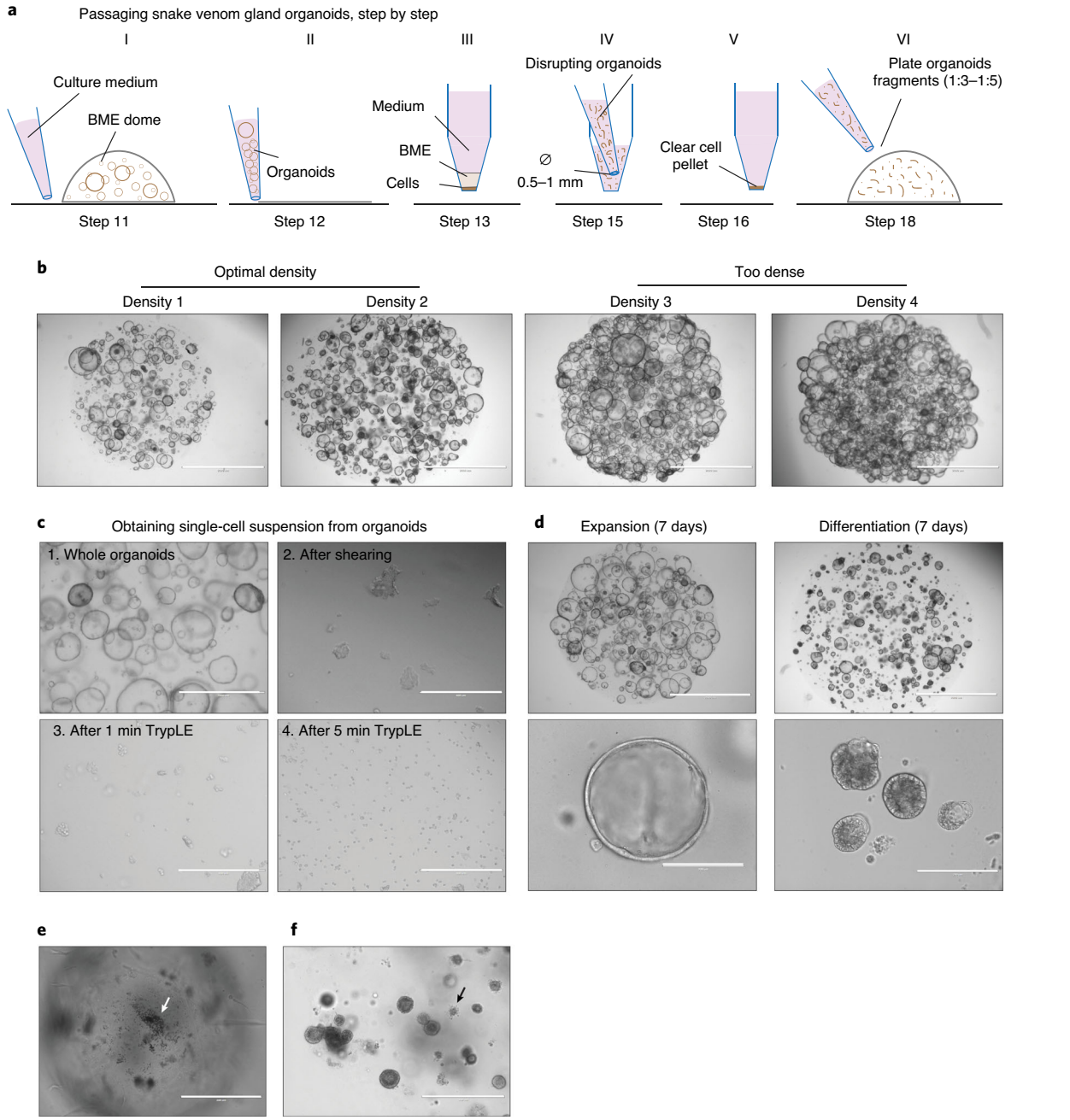

Fig. 3 | Setting up experiments with snake venom gland organoids. a, Schematic overview of passaging snake VGOs. Briefly, remove culture medium from organoids that are ready to be passaged (I). Take up organoids with the BME in ice-cold DMEM and pipette up and down to dissolve the BME (II). Centrifuge the cells (III). Mechanically disrupt the organoids into smaller pieces (IV). Centrifuge the small fragments (V). Plate obtained organoid fragments in fresh BME (VI). **b**, Representative brightfield images of four different plating densities of *A. lubricus* organoids. 1 and 2 are optimal densities, 3 and 4 are too dense. Circle is a 15 µL droplet. Scale bars, 2 mm. **c**, Time course of obtaining a single-cell suspension from organoids, after shearing and increasing time of dissociation using TrypLE. Scale bars, 400 µm. **d**, Representative brightfield images of *A. lubricus* organoids exposed to 7 d of expansion medium (left) or differentiation medium (right). Scale bars, upper panel 2 mm, lower panel 200 µm. **e**, Brightfield image of organoid with bacterial contamination. Bacteria cluster indicated with arrow. Scale bar, 400 µm. **f**, Brightfield image of organoid culture with poor viability. Arrow indicates death cells from organoid. Scale bar, 200 µm.

19 Resuspend the fragments in ice-cold BME (some DMEM can be left in the tube; the final BME concentration should be >60%). Plate in droplets of up to 20 µL (3 droplets per well) in a 24-well plate preheated to 32 °C (Fig. 3a, VI). Aim for a density as shown in Fig. 3b, density 1 or 2)
▲ **CRITICAL STEP** Keep BME on ice and work quickly to avoid solidification

20 Carefully flip the plate bottom-up and place it in an incubator at 32 °C. Leave to solidify for 20–30 min.

21 Place plate the right way up and add 500 µL of prewarmed expansion medium to each well.

22 Replace the medium every 3 d until the next passage or organoids are used for a further application.

▲ **CRITICAL STEP** After approximately one or two passages, organoids usually reach their stable growth speed, which is further influenced by the initial purity and density of the culture.

Downstream applications

23 Organoids can be passaged by repeating Steps 12–22, dissociated to single cells for FACS (option A), cryopreserved (option B) or differentiated and used to harvest toxins (option C).

(A) **Dissociation to single cells for FACS ● Timing 45 min (35 min hands-on)**

(i) Remove culture medium and resuspend BME dome in ice-cold DMEM by pipetting with a P1000 pipette.

(ii) Transfer resuspended organoids into a 15 mL Falcon tube and top up to 10 mL with ice-cold DMEM.

(iii) Centrifuge for 5 min at 4 °C at 300*g* to pellet organoids. The pellet should consist of 2 layers: A dense white pellet at the bottom which comprises the organoids and organoid fragments, as well as a transparent pellet of BME. If the cell pellet is mixed with the BME phase, remove DMEM, resuspend again in ice-cold DMEM and centrifuge until clear separation is reached.

(iv) Carefully aspirate DMEM and transparent layer of pellet.

(v) If organoids are larger than 200 µm, mechanically dissociate as described in Steps 16–18 (Fig. 3a).

(vi) Resuspend organoids in 1 mL of TrypLE containing 10 µM of Y-27632 preheated to 32 °C.

(vii) Incubate at 32 °C and repeatedly support digestion through mechanical shearing with a narrowed glass pipette or pipette tip every 2–3 min. Regularly assess fragment size by viewing the Falcon tube under a brightfield microscope. Stop immediately once ~90% of fragments are single cells (see Figs. 3c and 4.).

▲ **CRITICAL STEP** Close observation of the dissociation progress is essential. Depending on organoid number, size and differentiation state, dissociation times can vary between 2 and 10 min. Large quantities and concentrations of organoids require longer dissociation time. Similarly, smaller and more compact organoids (e.g., after differentiation) also require longer dissociation. Incomplete digestion leads to a substantial drop in retrieved single cell numbers, while superfluous digestion time rapidly impairs cell viability.

? TROUBLESHOOTING

(viii) Top up to 10 mL with digestion blocking buffer and centrifuge for 5 min at 4 °C and 300*g*.

(ix) Carefully aspirate supernatant and resuspend pellet in DMEM containing 10 µM of Y-27632. The resulting single cells can now be used for clonal outgrowth of organoids, quantitative seeding and genetic engineering. Further details of how we have previously used single cells are described in ref. [8].

(x) For downstream FACS applications, add 1 µg/mL DAPI for cell viability assessment to the cell suspension at this stage.

(xi) Pass suspension through the cell strainer cap of a FACS tube.

(xii) Proceed to preferred cell sorting or single cell sequencing protocol. Those we have used successfully are described in ref. [8].

(B) **Cryopreservation and thawing of organoids ● Timing 30 min to freeze (25 min hands-on), 45 min to thaw (25 min hands-on)**

▲ **CRITICAL** Small (~50 µm in diameter) organoids are preferable for cryopreservation as they yield the best recovery rates in our experience.

(i) *Freezing organoids*. For best results, shear small (~50 µm in diameter) organoids as described in Steps 12–22 2–3 d before freezing.

▲ **CRITICAL STEP** Avoid freezing single cells or large organoids, as these do not recover well on thawing.

(ii) Remove culture medium and resuspend BME dome(s) in ice-cold DMEM by pipetting with a P1000 pipette.

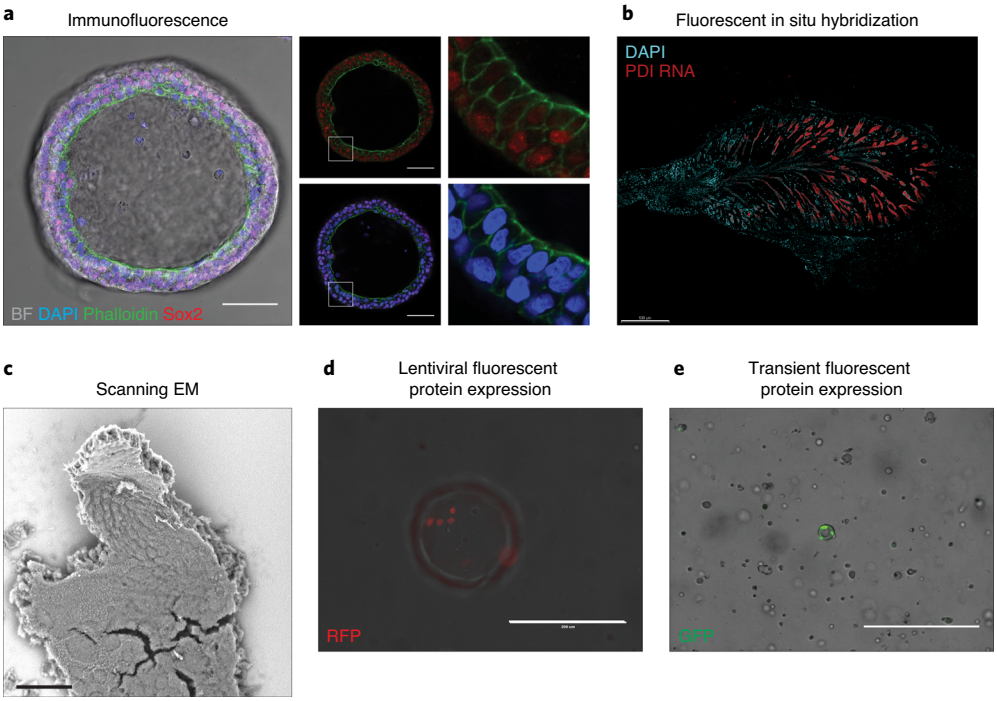

Fig. 4 | Analytical applications of snake venom gland organoids. a, Immunofluorescent image of *A. lubricus* organoids stained for DAPI (blue), F-actin (phalloidin - green) and Sox2 (red). Scale bar, 50 µm. **b**, Confocal microscopy image of fluorescent in situ hybridization of a late-embryonic venom gland. DAPI in blue and PDI (peptide disulfide isomerase, a protein important for toxin folding) RNA in red. Scale bar, 500 µm. **c**, Scanning electron microscopy (EM) image of an organoid fragment. Scale bar, 30 µm. **d**, Image of organoid expressing RFP from a lentiviral construct after transduction. Scale bar, 200 µm. **e**, Image of organoid transiently expressing GFP upon electroporation of construct. Scale bar, 400 µm. The methods used to obtain these results are detailed in Anticipated Results and the cited references.

(iii) Transfer resuspended organoids into a 15 mL Falcon tube and top up to 10 mL with cold DMEM.

(iv) Centrifuge for 5 min at 4 °C at 300*g* to pellet organoids. The pellet should consist of 2 layers: A dense white pellet at the bottom which comprises the organoids and organoid fragments, as well as a transparent pellet of BME. If the cell pellet is mixed with the BME phase, remove DMEM, resuspend again in ice-cold DMEM and centrifuge until clear separation is reached. Carefully aspirate DMEM and transparent layer of pellet.

(v) Resuspend organoids in 1 volume of DMEM. One well of a 12-well plate of organoids (~seven 15 µL droplets) should be resuspended in ~500 µL of DMEM.

(vi) Dropwise, add 1 volume of 2× freezing medium while constantly shaking the organoid tube.

(vii) Transfer organoids in the resulting 1× freezing medium into cryopreservation tubes (1 mL per tube is recommended) and transfer to a −80 °C freezer in a freezing container.

(viii) Allow the organoids to freeze overnight and transfer to liquid nitrogen for long-term storage.

PAUSE POINT Organoids can be frozen in liquid nitrogen indefinitely.

(ix) *Thawing of organoids*. Retrieve cryovial with organoids from liquid nitrogen and thaw in a water bath at 32 °C.

(x) Transfer thawed organoid suspension to an empty 15 mL Falcon tube immediately after the last frozen material is thawed.

CRITICAL STEP Do not leave the cells at 32 °C for longer than required for thawing.

(xi) Dropwise, top up to 10 mL with ice-cold DMEM under constant shaking of the Falcon tube.

CRITICAL STEP Add DMEM slowly to avoid the cells experiencing osmotic shock.

(xii) Centrifuge for 5 min at 4 °C at 300*g* to pellet organoids.

(xiii) Carefully aspirate and discard supernatant.

(xiv) Resuspend the fragments in ice-cold BME (some DMEM can be left in the tube; the final BME concentration should be >60%). Plate in droplets of ~15 µL (3 droplets per well) in a preheated 24-well plate (Fig. 3a, VI). Aim for a density as shown in Fig. 3b, density 1 or 2).

▲ **CRITICAL STEP** Keep BME on ice and work quickly to avoid solidification.

(xv) Carefully flip the plate bottom-up and place it in an incubator at 32 °C. Leave to solidify for 20–30 min.

(xvi) Flip plate back to correct orientation and add 500 µL of prewarmed expansion medium to each well.

(xvii) Refresh the medium every 3 d until the next passage (at which point you should follow Steps 12–22) or you are ready to move on to downsteam applications (see other Step 23 options).

? TROUBLESHOOTING

(C) **Differentiation of organoids and harvesting of toxins ● Timing 1 week for differentiation (30 min hands-on), plus 15 min hands-on time, 45 min for harvesting of toxins (15 min hands-on).**

▲ **CRITICAL** Organoids should be ~50 µm in average diameter to ensure optimal differentiation (this typically occurs 2–5 d after last passage).

(i) *Differentiation of organoids*. Remove expansion medium from the wells and wash with prewarmed DMEM.

▲ **CRITICAL STEP** Let DMEM incubate on top of organoids for ~15 min in an incubator at 32 °C to allow growth factors to diffuse from BME domes. Carry-over of stem cell factors will hamper differentiation.

(ii) Remove DMEM and add prewarmed differentiation medium.

(iii) Refresh differentiation medium the next day to remove any remaining stem cell factors and every 3 d thereafter. Within 7 d, the majority of cells should have differentiated to venom-producing cell types. The simplest indicator of successful differentiation is organoid morphology. Within a few days, organoids should appear less cystic, and should have thicker walls and be darker in color when observed under a brightfield microscope (Fig. 3d).

▲ **CRITICAL STEP** Once cells have achieved the appropriate morphology, they should secrete toxins into their lumen. The next steps can be used at appropriate timepoints to harvest toxins from the organoid lumen and intracellular vesicles. Proceed to the next step when you wish to harvest toxins.

▲ **CRITICAL STEP** The presence of venom-producing cells can be further monitored by carrying out qPCR on RNA extracted from cells at differing timepoints post transfer to differentiation medium.

? TROUBLESHOOTING

(iv) *Harvesting toxins from organoids*. Remove differentiation medium from the wells and wash with DMEM. Let DMEM incubate on top of organoids for ~15 min in an incubator at 32 °C to allow growth factors to diffuse from BME domes.

▲ **CRITICAL STEP** Growth factors could interfere with downstream assays such as functional toxin tests or proteomic analysis of venom composition.

(v) Remove DMEM from organoids and resuspend domes in 100 µL of ice-cold PBS per well of a 24-well plate.

(vi) Break open organoids and cells by sonicating organoids for 30 s in 15 cycles with a 30 s break between cycles.

▲ **CRITICAL STEP** On our experience these sonication conditions work for a variety of sizes and maturities of organoids.

(vii) Centrifuge for 10 min at 12,000*g* in a centrifuge precooled to 4 °C. Collect supernatant and snap-freeze on dry ice. Toxin supernatant can be stored at −80 °C for months. Avoid freeze–thaw cycles. Supernatant can be used for toxin characterization using ELISA and LC-MS or functional assays as described in refs. [12–14].

Troubleshooting

Troubleshooting advice can be found in Table 2.

Table 2 | Troubleshooting table

Step	Problem	Possible causes	Solution
1	Tissue falls apart/no organoid outgrowth	Decomposition due to slow dissection Extensive storage or shipment duration Freeze–thaw cycle or cytotoxins in gland	Proceed directly from dissection to seeding, avoid freezing, and add inhibitors of any species-specific cytotoxins
6	Tissue chunks remain intact/follicles do not dissociate	Starting material too large before collagenase treatment	Additional mechanical dissociation of the gland to avoid requirement for excessive incubation
11	Organoids do not grow into cystic spheres or lose morphology after first split	Unsuitable media conditions	Optimize culture medium for each species. Prepare fresh media with different batches of recombinant proteins/small molecules
	Bacterial outgrowth (Fig. 3e)	Bacterial contamination from the eggs/tissue	Additional ethanol washing of equipment and eggs. Analyze bacterial contamination and adjust antibiotics cocktail if needed.
	Cell death after culture initiation	Presence of residual cytotoxins from the venom gland	Additional washing of the dissociated glands before plating
23A(vii)	Poor single-cell dissociation	Starting material too large before TrypLE treatment	Additional mechanical dissociation of organoids before TrypLE treatment
		Some apoptotic cells releasing DNA, causing cell clumping	Addition of DNaseI to TrypLE to degrade extracellular DNA
23B(xvii)	Poor viability after thawing (Fig. 3f)	Organoids were too small or large during freezing	Freeze cystic organoids of <100 μm in size, ideally 2–3 d after freezing
		Osmotic shock during freezing or thawing procedure	Slowly add and dilute the freezing medium, making sure to shake constantly
23C(iii)	Poor differentiation	Residual growth factors preventing differentiation	Better washing and removing of expansion medium

Timing

Steps 1–11, Establishing a snake venom gland organoid line from adult or embryonic tissue: 3 h (2 h hands-on)

Steps 12–22, Passaging snake venom gland organoids: 45 min (25 min hands-on)

Downstream applications

Step 23A, Dissociation to single cells for FACS: 45 min (35 min hands-on)

Step 23B, Cryopreservation and thawing of organoids: 30 min to freeze (25 min hands-on), 45 min to thaw (25 min hands-on)

Step 23C, Differentiation of organoids and harvesting of toxins: 1 week for differentiation (30 min hands-on), 45 min for harvesting of toxins (15 min hands-on)

Anticipated results

Under optimal conditions, organoids can be derived from miniscule amounts of tissue (<10 mg) and expanded for years. Within days after plating, organoids should form as shown in Fig. 2. The venom gland-specific expansion medium provides positive selection for venom gland epithelial cells, resulting in loss of stromal cell types within the first 2–3 passages. The identity of the organoids can be confirmed by comparing toxin expression levels to original tissue and negative control organoids using qPCR (see below). Venom gland organoids in expansion medium produce enough toxin transcripts to confirm tissue identity in our experience.

When cultured in expansion medium, organoids should maintain a cystic shape (Fig. 2d) and be ready for an ~1:4 split every 1–3 weeks. For applications requiring higher numbers of venom-producing cells, differentiation medium should be applied, resulting in condensed organoid morphology within a week (Fig. 3d).

Snake VGOs can be used for several applications which were described in detail in previous publications[8,12–14]. We provide a short overview of the main assays and workflows below.

RNA-based methods are essential for organoid identity confirmation, assessment of differentiation and characterization of genetically modified organoids. RNA isolation for qPCR and RNA-sequencing applications can be performed directly from the domes of BME. Dissolving up to 100 µL drop volume of densely seeded organoids in 350 µL of buffer RLT (RNeasy Mini Kit, QIAGEN) or 1 mL TRIzol allows sufficient yield of RNA for all downstream applications. From this step onward, standard protocols for RNA isolation from tissue or cell lines can be followed[15]. For validation of differentiation, toxin family-wide primer pairs can be used in qPCR experiments[8].

Single cell RNA sequencing is becoming the gold standard for characterizing cellular heterogeneity in tissues, and can also be readily applied to organoid studies. Following dissociation of organoids to single cells (Steps 22–30), single-cell sequencing protocols[15,16] can be applied to organoid samples using methods that are analogous to those used on tissue samples.

While few species-specific antibodies exist for snakes, immunofluorescence (IF) is a powerful tool to obtain visual insights into the organization of tissue and organoids[8] (Fig. 4a). For immunofluorescence imaging, standard protocols for whole-mount organoid staining can be applied[17].

For immunohistochemical analysis, organoids can be collected using ice-cold DMEM and fixed in formalin for at least 2 h at room temperature. Downstream processing for paraffin embedding, sectioning and staining can be performed as described previously[9].

Given the lack of monoclonal antibodies for many reptile proteins, nucleic acid-based approaches such as fluorescence in situ hybridization (FISH) are necessary to probe the spatial patterns of gene expression. Standard in situ hybridization methods and novel approaches such as single molecule hybridization using RNAscope can be readily applied to snake VGOs[8] (Fig. 4b).

Snake VGOs are also amenable to higher resolution imaging using scanning electron microscopy[8] (Fig. 4c) and transmission electron microscopy[8].

The organoids can be genetically engineered using both lentiviral transduction[18] (Fig. 4d) and electroporation-based transfection[19] (Fig. 4e) protocols. Organoids are ideally dissociated into clumps of 5–10 cells using TrypLE before contact with the lentiviruses or plasmid DNA, respectively, for optimal transduction or transfection. Performing a single-cell dissociation and clonal outgrowth of lines (Steps 23–30) after genetic engineering is highly advisable, as well as antibiotic-based selection if applicable. The success of gene knock-out and fluorescent reporter knock-in can best be assessed by qPCR and fluorescent microscopy, respectively.

We anticipate that these methods will render a multitude of scientific questions addressable and that organoids will facilitate a deeper understanding of snake venom gland biology in the next years.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

References

1. Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262–265 (2009).
2. Kretzschmar, K. & Clevers, H. Organoids: modeling development and the stem cell niche in a dish. *Dev. Cell* **38**, 590–600 (2016).
3. Clevers, H. Modeling development and disease with organoids. *Cell* **165**, 1586–1597 (2016).
4. Gutierrez, J. M. et al. Snakebite envenoming. *Nat. Rev. Dis. Primer* **3**, 17063 (2017).
5. Sells, P. G., Hommel, M. & Theakston, R. D. G. Venom production in snake venom gland cells cultured in vitro. *Toxicon* **27**, 1245–1249 (1989).
6. Carneiro, S. M. et al. Venom production in long-term primary culture of secretory cells of the Bothrops jararaca venom gland. *Toxicon* **47**, 87–94 (2006).
7. Yamanouye, N. et al. Long-term primary culture of secretory cells of Bothrops jararaca venom gland for venom production in vitro. *Nat. Protoc.* **47**, 87–94 (2007).
8. Post, Y. et al. Snake venom gland organoids. *Cell* **180**, 233–247.e21 (2020).
9. Sato, T. et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* **141**, 1762–1772 (2011).
10. Boj, S. F. et al. Organoid models of human and mouse ductal pancreatic cancer. *Cell* **160**, 324–338 (2015).
11. Schutgens, F. et al. Tubuloids derived from human adult kidney and urine for personalized disease modeling. *Nat. Biotechnol.* **37**, 303–313 (2019).

12. Nicolas, J. et al. Detection of marine neurotoxins in food safety testing using a multielectrode array. *Mol. Nutr. Food Res.* **58**, 2369–2378 (2014).
13. Whiteley, G. et al. Defining the pathogenic threat of envenoming by South African shield-nosed and coral snakes (genus *Aspidelaps*), and revealing the likely efficacy of available antivenom. *J. Proteomics* **198**, 186–198 (2019).
14. Slagboom, J. et al. High throughput screening and identification of coagulopathic snake venom proteins and peptides using nanofractionation and proteomics approaches. *PLoS Negl. Trop. Dis.* **14**, e0007802 (2020).
15. Muraro, M. J. et al. A single-cell transcriptome atlas of the human pancreas. *Cell Syst* **3**, 385–394.e3 (2016).
16. Baran-Gale, J., Chandra, T. & Kirschner, K. Experimental design for single-cell RNA sequencing. *Brief. Funct. Genomics* **17**, 233–239 (2018).
17. Dekkers, J. F. et al. High-resolution 3D imaging of fixed and cleared organoids. *Nat. Protoc.* **14**, 1756–1771 (2019).
18. Drost, J., Artegiani, B. & Clevers, H. The generation of organoids for studying WNT signaling. *Methods Mol. Biol.* **1481**, 141–159 (2016).
19. Fujii, M., Matano, M., Nanki, K. & Sato, T. Efficient genetic engineering of human intestinal organoids using electroporation. *Nat. Protoc.* **10**, 1474–1485 (2015).
20. Lu, Y. et al. Avian-induced pluripotent stem cells derived using human reprogramming factors. *Stem Cells Dev.* **21**, 394–403 (2012).
21. Peng, L. et al. Generation of stable induced pluripotent stem-like cells from adult zebra fish fibroblasts. *Int. J. Biol. Sci.* **15**, 2340–2349 (2019).
22. Pierzchalska, M., Panek, M., Czyrnek, M. & Grabacka, M. The three-dimensional culture of epithelial organoids derived from embryonic chicken intestine. *Methods Mol. Biol.* **1576**, 135–144 (2019).

Acknowledgements

We thank A. de Graaff and the Hubrecht Imaging Centre (HIC) for microscopy assistance; B. Ponsioen for providing a lentiviral H2B-RFP construct; and local snake breeders as well as W. Getreuer and SERPO for donating venom gland material.

Data availability

All previously unpublished data is included in the figures. Raw image files are available from the corresponding author upon request.

Competing interests

H.C. is inventor on multiple patents held by the Dutch Royal Netherlands Academy of Arts and Sciences that cover organoid technology: PCT/NL2008/050543, WO2009/022907; PCT/NL2010/000017, WO2010/090513; PCT/IB2011/002167, WO2012/014076; PCT/IB2012/052950, WO2012/168930;PCT/EP2015/060815, WO2015/173425; PCT/EP2015/077990, WO2016/083613; PCT/EP2015/077988, WO2016/083612; PCT/EP2017/054797,WO2017/149025; PCT/EP2017/065101, WO2017/220586; PCT/EP2018/086716; and GB1819224.5. H.C.'s full disclosure is given at https://www.uu.nl/staff/JCClevers/.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41596-020-00463-4.

Correspondence and requests for materials should be addressed to H.C.

Peer review information *Nature Protocols* thanks C. Caldeira, J. Calvete and C. M. Trim for their contribution to the peer review of this work.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 22 April 2020; Accepted: 12 November 2020;
Published online: 27 January 2021

Related links
Key reference using this protocol
Post, Y. et al. *Cell* **180**, 233–247.e21 (2020): https://doi.org/10.1016/j.cell.2019.11.038

nature research

Corresponding author(s): Hans Clevers

Last updated by author(s): Jul 1, 2020

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☒	☐ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☒	☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐ A description of all covariates tested
☒	☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☒	☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Leica LAS X Version 1.1
Data analysis	ImageJ (Fiji, Version 1.51n), Microsoft Excel 2016, Leica LAS X Version 1.1

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Not applicable

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No sample size calculation was performed as not statistical analysis was performed in this protocols paper.
Data exclusions	No data exclusion was performed as not statistical analysis was performed in this protocols paper.
Replication	All figures display representative images of experiments that were replicated at least 2 times.
Randomization	No randomization was performed as not statistical analysis was performed in this protocols paper.
Blinding	No blinding was performed as not statistical analysis was performed in this protocols paper.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Rabbit anti-SOX2; Millipore; cat no. AB5603; RRID:AB_2286686 Rabbit anti-B-catenin; Santa Cruz; cat no. # sc-7199; RRID: AB_634603 Rabbit Anti-β-Tubulin; Santa Cruz; cat no. # sc-9104; RRID: AB_2241191 Phalloidin-Alexa 647; Thermo Scientific; cat no. A22287 RRID: AB_2620155
Validation	Antibodies had not previously been validated on Aspidelaps lubricus organoids.