**Derivation of snake venom gland organoids for *in vitro* venom production**

Jens Puschhof1,2,\*, Yorick Post1,2,\*, Joep Beumer1,2,\*, Harald M. Kerkkamp3,4, Matyas Bittenbinder3,5, Freek J. Vonk3, Nicholas R. Casewell6, Michael K. Richardson4 and Hans Clevers1,2,🖂

1 Hubrecht Institute, Royal Netherlands Academy of Arts and Sciences (KNAW) and UMC Utrecht, 3584 CT Utrecht, the Netherlands

2 Oncode Institute, Hubrecht Institute, 3584 CT Utrecht, the Netherlands

3 Naturalis Biodiversity Center, 2333 CR Leiden, the Netherlands

4 Institute of Biology Leiden, Department of Animal Science and Health, 2333 BE 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, De Boelelaan 1085, 1081 HV Amsterdam

6 Centre for Snakebite Research & Interventions, Department of Tropical Disease Biology, Liverpool School of Tropical Medicine, Liverpool L3 5QA, UK

\* Co-first author

🖂Lead Contact

Correspondence: h.clevers@hubrecht.eu

**Abstract**

More than 400,000 people each year suffer adverse effects following bites from venomous snakes. However snake venom also provides a rich resource of bioactive molecules for drug development. Milking snakes is the most common method to obtain antivenom. Safer, alternative methods to produce venom could facilitate production of both antivenom and novel therapeutics. This protocol describes the generation, maintenance and 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 of several snake species and can be maintained long-term (we have cultured some for more than 2 years). We have successfully used late stage embryos from eggs and glands from 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 into single cells, cryopreserve and differentiate into toxin producing organoids. We also provide guidance on downstream assays that can be implemented, 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 on venom production, differentiation and cellular heterogeneity.

**Introduction**

Since their development 10 years ago, adult stem cell-derived organoids have been established from most epithelial tissues of humans and mice1,2. Their ability to maintain cellular heterogeneity, self-organize into three dimensional structures and be maintained long-term have made organoids invaluable tools in biomedical research3.

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 year4 but also represents a rich resource of bioactive molecules for drug development. For these reasons, many efforts are being made to understand venom production and function, including through the derivation of tissue models of the snake venom gland5–7. While previous *in vitro* models of the snake venom gland have shown the ability to derive cell lines and suspension cultures, broad utility of these models has been hampered by the short lifespan of models or lack of representation of the tissue complexity.

This protocol aims to address this need by providing detailed instructions for 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 families8. 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 this paper underlines the validity of snake venom gland organoids (VGOs) as an *in vitro* model of the venom 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 previously published human organoid systems9,10. Whilst similar to human pancreatic organoid medium, the snake VGO media comprises a unique mixture of mammalian growth factors which have been optimized for expansion and differentiation of VGOs8. Durations and 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 preferable to using this protocol, particularly if cost is a consideration, or rapid large scale production of a specific venom is required.

Crude venom can be obtained by milking snakes. Currently venom derived from milking snakes yields larger amounts and guarantees broad coverage of toxins in the venom. However, we anticipate that producing venom from organoids could be preferable for applications that require scalability or if the precise composition is important, as modification of individual toxins is easier.

Previously developed snake venom gland cell lines permit modelling of some characteristics of the original organ. These cultures, grown in suspension as single cells5 or follicular structures6,7, can be grown for months to years at lower costs than organoids and produce some venom. An advantage of organoids is that adult stem cells remain present in the culture long-term, 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 used8 which facilitates more targeted differentiation of cell types, enabling 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 10 different species, demonstrating its robustness and more general applicability. Genetic modification, targeted differentiation of specific cell types and manipulation of the production of specific venoms has 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 interactions with surrounding muscle and nerve cells cannot be modelled. The organoids maintain regional characteristics8, so the starting material should be carefully considered to ensure the appropriate part of the venom gland is modelled. This characteristic can also be advantageous, for example we applied this system to study the cellular and regional heterogeneity of the venom gland8.

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 may pave the way to the production of optimized toxins in ideally suited cell types.

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).

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 (Lu et al., 2011). A similar strategy was recently used to generate iPSCs from fish fibroblasts (Peng et al., 2019).

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 (Pierzchalska et al).

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

**Experimental Design**

Snake venom gland organoids can either be shared in cryopreserved form (in which case you should start the procedure at step 23Bx) or be established from a new biopsy of an embryonic or adult snake (by starting the procedure at step 1A or B, respectively). The euthanization of adult snakes was described in detail before7 and is not included here. In our experience, suitable biopsies can be obtained from snakes that died of natural courses within a day of their death. Nevertheless, when using adult snake-derived biopsies, storage and transport times should be minimized. Extensive washing may be necessary if cytotoxins are abundant in the venom to avoid decreased viability of 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 cultures derived from biopsies from adult snakes. We have previously found a similar to adult toxin expression profile in late stage embryonic biopsy cultures8, with a negligible effect of cytotoxins on cell viability, thus we recommend 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 may be a 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 urine11.

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

**Table 1:** Overview on species, starting material and time in culture of snake venom gland organoids.

|  |  |  |
| --- | --- | --- |
| **Species** | **Adult/embryo** | **Time in culture** |
| *Aspidelaps lubricus cowlesi* | Embryo | 24 months |
| *Naja pallida* | Adult | 1 month |
| *Naja annulifera* | Embryo | 1 month |
| *Naja nivea* | Adult | 6 months |
| *Naja atra* | Embryo | 3 months |
| *Echis ocellatus* | Adult | 1 month |
| *Deinagkistrodon acutus* | Adult | 1 month |
| *Crotalus atrox* | Adult | 1 month |
| *Bitis arietans* | Adult | 3 months |
| *Naja haje* | Embryo | 1 week |

In our experience, organoids grow at a wide variety of speeds. For our studies to date we only received tissue once or twice 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 in duration and different batches of media were used. It is thus hard to pinpoint the exact influence of individual factors on the rate of growth. Users should thus expect organoids from different source material to grow at differing speeds. We recommend moving to subsequent stages of the procedure when organoids reach the indicated size (see relevant steps in the procedure) rather than at a particular timepoint.

Organoid morphology, split ratio and toxin gene expression best determine whether organoids are successfully established. In the expansion medium used for establishing and propagating organoids, they should grow as spheres as shown in **Fig. 3b**. More dense 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 stable growth phase (usually 2 passages after establishing), it should be split by a factor of ~1:3 every 7-14 days. Significantly lower expansion speed can indicate suboptimal media conditions. Finally, the identity of the grown organoids should be established as early as possible using quantitative real-time PCR for cell type markers. Toxin genes are well-suited to distinguish venom gland organoids from any other tissue. As reference, RNA extracted from primary venom gland tissue and non-venom gland tissue should be assessed. Any expression significantly over the non-venom gland tissue level is indicative of venom gland organoids.

Many of the proposed applications presented in this protocol require venom gland organoid 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 days. Quantitative real-time PCR for toxin genes can be used to track differentiation dynamics and determine the ideal time point for differentiation towards production of specific toxins. Upon differentiation, organoids stop proliferating and they 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 **Figure 1**.

**Materials**

**Biological starting material**

Dead snake. Detailed guidelines for the sacrifice of snakes is provided in8. *CAUTION*: Handling venomous snakes is extremely dangerous and should only be performed by trained experts. Make sure approval from the relevant animal experiment board is obtained. It is also important that 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 the snake is humanely euthanized according to the recommendations of the relevant ethics authority. We obtained approval for our studies published in 8 from the following institutions: *Crotalus atrox* from 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, Naja 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. *Naja atra, Naja annulifera* and *Aspidelaps lubricus cowlesi* embryonic material was obtained from local breeders in The Netherlands. Local animal research law (Wet op Dierproeven, WOD, artikel 1b lid 5a) enables the use of embryonic reptile material for research purposes.

**Reagents**

**Chemicals, Peptides, Recombinant Proteins, Antibodies and Kits**

* Advanced 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. R001
* R-spondin conditioned medium; U-Protein Express; cat no. N002
* Human EGF; Peprotech; cat no. AF-100-15
* A83-01; Tocris; cat no. 2939
* Prostaglandin E2; Tocris; cat no. 2296
* Gastrin I; Tocris; cat no. 3006
* Human 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
* Azithromycin dehydrate; Sigma-Aldrich; cat no. PZ0007
* FBS; Thermo Scientific; cat no. 16140071
* 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 (https://scicrunch.org/resolver/RRID:AB\_2629482); dilution 1:1000
* Rabbit anti-SOX2; Millipore; cat no. AB5603; RRID:AB\_2286686 (https://scicrunch.org/resolver/RRID:AB\_2286686); dilution 1:100
* Rabbit anti-B-catenin; Santa Cruz; cat no. # sc-7199; RRID: AB\_634603 (https://scicrunch.org/resolver/RRID:AB\_2286686); dilution 1:100
* Rabbit Anti-β-Tubulin; Santa Cruz; cat no. # sc-9104; RRID: AB\_2241191 (https://scicrunch.org/resolver/RRID: AB\_2241191)
* Phalloidin-Alexa 647; Thermo Scientific; cat no. A22287 RRID: AB\_2620155 (https://scicrunch.org/resolver/RRID: AB\_2620155); dilution 1:1000
* 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 ; QIAGE;N cat no. 74104

**EQUIPMENT**

Falcon tubes 15 ml

Falcon tubes 50 ml

5 ml polystyrene round-bottom tube with cell-strainer caps (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% CO2 incubator (at 32°C)

32°C shaking platform

Biosafety cabinet

**Reagent setup**

***Snake venom gland organoid expansion medium***

For establishing and maintenance of VGOs, expansion culture medium is used.

To make expansion medium, supplement AdDMEM/F12 with B-27 Supplement (1x), Glutamax (1x), HEPES (1x), 100 U/mL Penicillin-Streptomycin, 100 µg/mL Primocin, 1.25mM N-acetylcysteine, 10 mM Nicotinamide. The following growth factors should be added: 2% Noggin conditioned medium (vol/vol), 2% Rspo3 conditioned medium (vol/vol), 50 ng/mL EGF, 0.5 µM A83-01, 1 µM PGE2, 100 nM Gastrin, 100 ng/mL FGF10.

CRITICAL Depending on species, adding 1 µM Forskolin (FSK) may have a beneficial effect (we have found this to be beneficial for *Naja nivea*).

CRITICAL STEP After every step in which the organoids are dissociated to single cells, 10 µM Y-27632 should be added for at least 5 days or until cystic organoids have formed again, to prevent anoikis.

*CRITICAL STEP*: The organoid medium should be used within a month after preparation and stored at 4°C. It can be shipped frozen or liquid and used within the same timeframe after thawing. Avoid freeze-thaw cycles.

***Antibiotics cocktail***

During establishment of an organoid culture and in case of bacterial contaminations, 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 STEP*: The organoid medium with added antibiotics should be used within a month after preparation and stored at 4°C. It can be shipped frozen or liquid 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 venom gland organoids. As for expansion medium, to make differentiation medium, supplement AdDMEM/F12 with Glutamax (1x), HEPES (1x), 100 U/mL Penicillin-Streptomycin, 100 µg/mL Primocin, 1.25 mM N-acetylcysteine, 10 mM Nicotinamide and 1 µM PGE2.

*CRITICAL STEP*: The organoid medium should be used within a month after preparation and stored at 4°C. It can be shipped frozen or liquid and used within the same timeframe after thawing. Avoid freeze-thaw cycles.

***Organoid freezing medium***

To cryopreserve snake venom gland organoids, a 2x freezing medium stock is prepared. For 2x freezing medium, add 1 volume of DMSO to 4 volumes of FBS. Freezing stock medium can be stored at 4°C for months.

*CRITICAL STEP*: The organoid medium should be used within a month after preparation and stored at 4°C. It can be shipped frozen or liquid 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 STEP*: The blocking buffer should be used within three months after preparation and stored at 4°C. After addition of Y-27632, the medium should be used within a month. It can be shipped frozen or liquid 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 at −20°C for all of the following.

*B-27 supplement*

Provided as 50× stock solution. Store at −20°C.

*N-Acetylcysteine*

Dissolve 81.5 mg per ml H2O to prepare a 400× 500mM stock solution. Store at −20°C.

*Nicotinamide*

Dissolve 1.2 g in 10 ml PBS to prepare a 100× 1M stock solution. Store at −20°C.

*Noggin conditioned medium*

Provided as a 50x stock solution. Store at -20°C.

*R-spondin conditioned medium*

Provided as a 50x stock solution. Store at -20°C.

*Human EGF*

Dissolve 1 mg in 2 ml PBS + 0.1% BSA to prepare a 10,000× 0.5mg/ml stock solution. Store at −20°C.

*A83-01*

Dissolve 2 mg in 950 μl DMSO to obtain a 5mM 50,000× stock solution. Store at −20°C.

*PGE2*

Dissolve 10 mg in 2.84 ml DMSO to prepare a 10,000× 10mM stock solution. Store at −20°C.

*FGF-10*

Dissolve 500 μg in 5 ml PBS + 0.1% BSA to prepare a 10,000× 0.1 mg/ml stock solution. Store at −20°C.

*Y-27632*

Dissolve 50 mg in 1.5 ml H2O to prepare a 10,000× 100mM stock solution. Store at −20°C.

*Collagenase*

Dissolve 20 mg Collagenase in 1 ml of adDMEM/F12 to make a 20 mg/ml stock solution. Store at -20°C.

*Forskolin*

Dissolve the powder in DMSO to prepare a 10,000x 10 mM stock solution. Store at -20°C.

*Gastrin I*

Dissolve the powder in PBS + 0.1% BSA to prepare a 10,000x 100 µM stock solution. Store at -20°C.

*Gentamicin*

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

*Ciprofloxacin*

Dissolve in DMSO to a 10,000x stock with a concentration of 25 mg/ml. Store at -20°C.

*Erythromycin*

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

*Azithromycin*

Dissolve in DMSO to a 100,000x 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*: 2h hands-on time, 3h in total)**

*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 8.

CRITICAL Detailed guidelines for the sacrificing of snakes and further description of how to dissect the venom gland is also provided in 7.

1. Dissect venom glands from a freshly sacrificed snake as described in option A for late-stage embryos or option B for adult snakes.
2. 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.

1. 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.

PAUSEPOINT Dissected glands can be kept for up to one day 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*

1. Using scalpels, remove connective tissue, fat and other tissue parts as much as possible (**Fig. 2b**). If available, a stereomicroscope should be used to guide tissue identification in 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.
2. 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.*

1. Further cut the venom gland into small pieces (ideally <1 mm) with scalpels.
2. Wash pieces 3 times with DMEM containing the antibiotics cocktail and 100 µg/mL Primocin.
3. 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 minutes at 32°C under constant rocking (~120 RPM on a tube rocker). Repeatedly harvest ~20 µl of solution and assess tissue digestion under a brightfield microscope (start checking after 15 minutes and repeat every 5-10 minutes). Stop the digestion once follicular structures become apparent and start to dissociate by proceeding with the next step.
*CRITICAL STEP:* Avoid temperatures above 32°C in the digestion process. While these can be beneficial to enzyme activity, if the cells produce a heat shock response, viability can be dramatically reduced (as shown in 8).
*TROUBLESHOOTING*
4. Top up falcon tube to 10 ml with DMEM containing the antibiotics cocktail and 100 µg/mL Primocin and centrifuge for 5 minutes at 4°C and 300 x 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.

1. Aiming for a density of ~150 organoid structures per drop of 15 µl (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.

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*CRITICAL STEP:* Keep BME on ice and work quickly to avoid solidification prior to plating.

1. 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 minutes.
2. Flip plate the right way up and add 500 µl of pre-warmed expansion medium with 10 µM Y-27632 to each well of a 24-well plate (2 ml per well in 6-well plate, 1 ml per well in 12-well plate, 300 µl per well in a 48-well plate).
3. Refresh medium every 3 days, depending on organoid growth. Once cystic organoids with a size >200 µm in diameter are formed (usually within 2-10 days, **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*: 25 minutes hands-on time, 45 minutes total)**

*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 venom gland organoids being grown in expansion medium to confirm the identity of the organoids by qPCR. Thus, organoids can be further characterised by qPCR at this stage.

1. Remove culture medium and resuspend BME dome(s) in cold DMEM by pipetting with a P1000 pipette.
2. Transfer resuspended organoids into 15 ml falcon tube and top up to 10 ml with cold DMEM (**Fig. 3a-II**).
3. Centrifuge for 5 minutes at 300 x 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 (**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.

1. Carefully aspirate DMEM and transparent layer of pellet.
*CRITICAL STEP*: The cell pellet may 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.
2. 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.
3. Top up tube containing organoids to 10 ml with cold DMEM and centrifuge at 4°C at 300 x g for 5 minutes to pellet organoid fragments (**Fig. 3a-V**).
4. Aspirate DMEM supernatant.
5. 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

1. Carefully flip the plate bottom-up and place it in an incubator at 32°C. Leave to solidify for 20-30 minutes.
2. Place plate the right way up and add 500 µl of prewarmed expansion medium to each well.
3. Replace the medium every 3 days until the next passage or organoids are used for a further application.

CRITICAL STEP After ~1-2 passages organoids usually reach their stable growth speed, which is further influenced by the initial purity and density of the culture.

**Downstream applications**

1. Organoids can be passaged, by repeating steps 12-22 long term or 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*: 35 minutes hands-on time, 45 minutes total)**

1. Remove culture medium and resuspend BME dome in ice cold DMEM by pipetting with a P1000 pipette.
2. Transfer resuspended organoids into a 15 ml falcon tube and top up to 10 ml with ice cold DMEM.
3. Centrifuge for 5 minutes at 4°C at 300 x 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.
4. Carefully aspirate DMEM and transparent layer of pellet.
5. If organoids are larger than 200 µm, mechanically dissociate as described in steps 16-18 (**Fig. 3a**).
6. Resuspend organoids in 1 ml of TrypLE containing 10 µM of Y-27632 preheated to 32°C.
7. Incubate at 32°C and repeatedly support digestion through mechanical shearing with a narrowed glass pipette or pipet tip every 2-3 minutes. Regularly assess fragment size by viewing the falcon tube under a brightfield microscope. Stop immediately once ~90% of fragments are single cells (**see Fig. 3c-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 minutes. Large quantities and concentrations of organoids require longer dissociation time. Similarly, smaller and 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*
8. Top up to 10 ml with digestion blocking buffer and centrifuge for 5 minutes at 4°C and 300 x g.
9. 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 what we have previously used single cells for are described in 8.
10. For downstream FACS applications, add 1 µg/ml DAPI for cell viability assessment to the cell suspension at this stage.
11. Pass suspension through the cell strainer cap of a FACS tube.
12. Proceed to preferred cell sorting or single cell sequencing protocol. Those we have used successfully are described in 8.

**B) Cryopreservation and thawing of organoids (*TIMING:* 30 minutes in total to freeze (25 minutes hands-on time), 45 minutes to thaw in total (25 min hands-on time))**

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

1. **Freezing organoids.**  For best results, shear small sized (~50 µm in diameter) organoids as described in steps 12-22 2-3 days before freezing.
*CRITICAL STEP:* Avoid freezing single cells or large organoids, as these do not recover well on thawing.
2. Remove culture medium and resuspend BME dome(s) in ice cold DMEM by pipetting with a P1000 pipette.
3. Transfer resuspended organoids into a 15 ml falcon tube and top up to 10 ml with cold DMEM.
4. Centrifuge for 5 minutes at 4°C at 300 x 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.
5. Carefully aspirate DMEM and transparent layer of pellet.
6. 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.
7. Dropwise add 1 volume of 2x freezing medium while constantly shaking the organoid tube.
8. Transfer organoids in the resulting 1x freezing medium into cryopreservation tubes (1 ml per tube is recommended) and transfer to a -80°C freezer in a freezing container.
9. Allow the organoids to freeze overnight and transfer to liquid nitrogen for long-term storage.

PAUSEPOINT Organoids can be frozen in liquid nitrogen indefinitely.

1. **Thawing of organoids .** Retrieve cryovial with organoids from liquid nitrogen and thaw it in a water bath at 32°C.
2. 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.
3. 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.
4. Centrifuge for 5 minutes at 4°C at 300 x g to pellet organoids.
5. Carefully aspirate and discard supernatant.
6. 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.

1. Carefully flip the plate bottom-up and place it in an incubator at 32°C. Leave to solidify for 20-30 minutes.
2. Flip plate back to correct orientation and add 500 µl of prewarmed expansion medium to each well.
3. Refresh the medium every 3 days until the next passage (at which point you should follow steps 12-22) or application (see other step 23 options).

*TROUBLESHOOTING*

**C) Differentiation of organoids and harvesting of toxins (*TIMING*: 30 minutes hands-on time, 1 week in total for differentiation, plus 15 minutes hands-on time, 45 minutes total for harvesting of toxins )**CRITICAL Organoids should be ~50 µm in average diameter to ensure optimal differentiation (this typically occurs 2-5 days after last passage).

1. ***Differentiation of organoids.*** Remove expansion medium from the wells and wash with pre-warmed DMEM.
*CRITICAL STEP:* Let DMEM incubate on top of organoids for ~15 minutes in an incubator at 32°C to allow growth factors to diffuse from BME domes. Carry-over of stem cell factors will hamper differentiation.
2. Remove DMEM and add pre-warmed differentiation medium.
3. Refresh differentiation medium the next day (to remove any remaining stem cell factors) and after every 3 days. Within 7 days, 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 colour when observed under a brightfield microscope (**Fig. 3d**). CRITICAL STEP Once cells have appropriate morphology, they should secrete toxins into their lumen. The next steps can be used at appropriate time points to harvest toxins from the organoid lumen and intracellular vesicles. Proceed to the next step when you wish to harvest toxins.
4. CRITICAL STEP The presence of venom-producing cells can be further checked for by carrying out qPCR on RNA extracted from cells at differing time points post transfer to differentiation medium.

TROUBLESHOOTING

1. **Harvesting toxins from organoids.** Remove differentiation medium from the wells and wash with DMEM.Let DMEM incubate on top of organoids for ~15 minutes 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.

1. Remove DMEM from organoids and resuspend domes in 100 µl of ice cold PBS per well of a 24-well plate.
2. Break open organoids and cells by sonicating organoids for 30 seconds in 15 cycles with a 30 second break between cycles.

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

1. Centrifuge for 10 minutes at 12,000 x g in a centrifuge pre-cooled 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 12–14.

**Troubleshooting**

**See Table 2 for troubleshooting guidance.**

**Table 2: Troubleshooting guidance**

|  |  |  |  |
| --- | --- | --- | --- |
| **Step** | **Problem** | **Possible causes** | **Solution** |
| 1/2/11 | Tissue falls apart / no organoid outgrowth | 1. Decomposition due to slow dissection.
2. Extensive storage or shipment duration.
3. 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 free up | 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. |
| 11 | Bacteria 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. |
| 11 | Cell death after culture initiation | Remaining cytotoxins from the venom gland | Additional washing of the dissociated glands before plating |
| A-vii | Poor single cell dissociation | 1. Starting material too large before TrypLE treatment.
2. Some apoptotic cells releasing DNA, causing cell clumping
 | 1. Additional mechanical dissociation of organoids before TrypLE treatment.
2. Addition of DNaseI to TrypLE to degrade extracellular DNA.
 |
| B-xviii | Poor viability after thawing (**Fig. 3f**) | 1. Organoids were too small or large during freezing
2. Osmotic shock during freezing or thawing procedure
 | 1. Freeze cystic organoids of < 100 µm in size, ideally 2-3 days after freezing
2. Slowly add and dilute the freezing medium, make sure to shake constantly.
 |
| C-iii | Poor differentiation | Remaining growth factors preventing differentiation | Better washing and removing of expansion medium |

**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 Figure 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 a ~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 venom gland organoids can be used for several applications which were described in detail before8,12–14. 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 followed15. For validation of differentiation, toxin family-wide primer pairs can be used in qPCR experiments8.

Single cell RNA sequencing is becoming the gold standard for characterizing cellular heterogeneity in tissues, and can also readily be applied to organoid studies. Following dissociation of organoids to single cells (steps 22-30), single cell sequencing protocols15,16 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 organoids8 (**Fig. 4a**). For immunofluorescence imaging, standard protocols for whole-mount organoid staining can be applied17.

For immunohistochemical analysis, organoids can be collected using ice cold DMEM and fixed in formalin for at least 2 hours at room temperature. Downstream processing for paraffin embedding, sectioning and staining can be performed as described previously9.

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 venom gland organoids8 (**Fig. 4b**)

Snake venom gland organoids are also amenable to higher resolution imaging using scanning electron microscopy8 (**Fig. 4c**) and transmission electron microscopy8.

The organoids can be genetically engineered using both lentiviral transduction18 (**Fig. 4d)** and electroporation-based transfection19 (**Fig. 4e**) protocols. Organoids are ideally dissociated into clumps of 5-10 cells using TrypLE before contacting 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.

**FIGURE LEGENDS**

**Figure 1 – Overview of working with snake venom gland organoids.** Top: a schematic representation of the steps from establishing organoids from primary venom gland tissue, passaging and expanding organoids. Middle: the possibility to differentiate the organoids for higher venom production. Bottom: the possible research applications with organoids in expansion medium and/or differentiation medium.

**Figure 2 - Establishment of snake venom gland organoids from primary tissue.** (**a**) Isolation and dissection of late-stage embryonic (~ 5 days before hatching) *Aspidelaps lubricus* to obtain venom gland tissue in sterile conditions. (**b**) Complete late-embryonic venom gland from *Naja annulifera* after removal of muscle and connective tissue. Scale bar increments are 1 mm. (**c**) *Naja annulifera* venom gland (from b) after separation into proximal (P, ductal), middle (M) and distal (D) part 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 approximately 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 *Aspidelaps lubricus* venom gland cells in BME (passage 0) from day 0 to day 10. Scale bars, 1 mm.

**Figure 3 - Setting up experiments with snake venom gland organoids.** (**a**) A schematic overview of passaging snake venom gland organoids. Briefly, remove culture medium from organoids that are ready to be passaged (*I*). Take op organoids with the BME in ice cold DMEM and pipet 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 *Aspidelaps lubricus* organoids. 1 and 2 are optimal densities, 3 and 4 are too dense. Circle is 15 uL 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 *Aspidelaps lubricus* organoids exposed to seven days 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 indicated death cells from organoid. Scale bar, 200 µm.

**Figure 4 - Analytical applications of snake venom gland organoids.** (**a**) Immunofluorescent image of *Aspidelaps 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 is 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.

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**Data availability statement**

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

**Competing interest**

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/.

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