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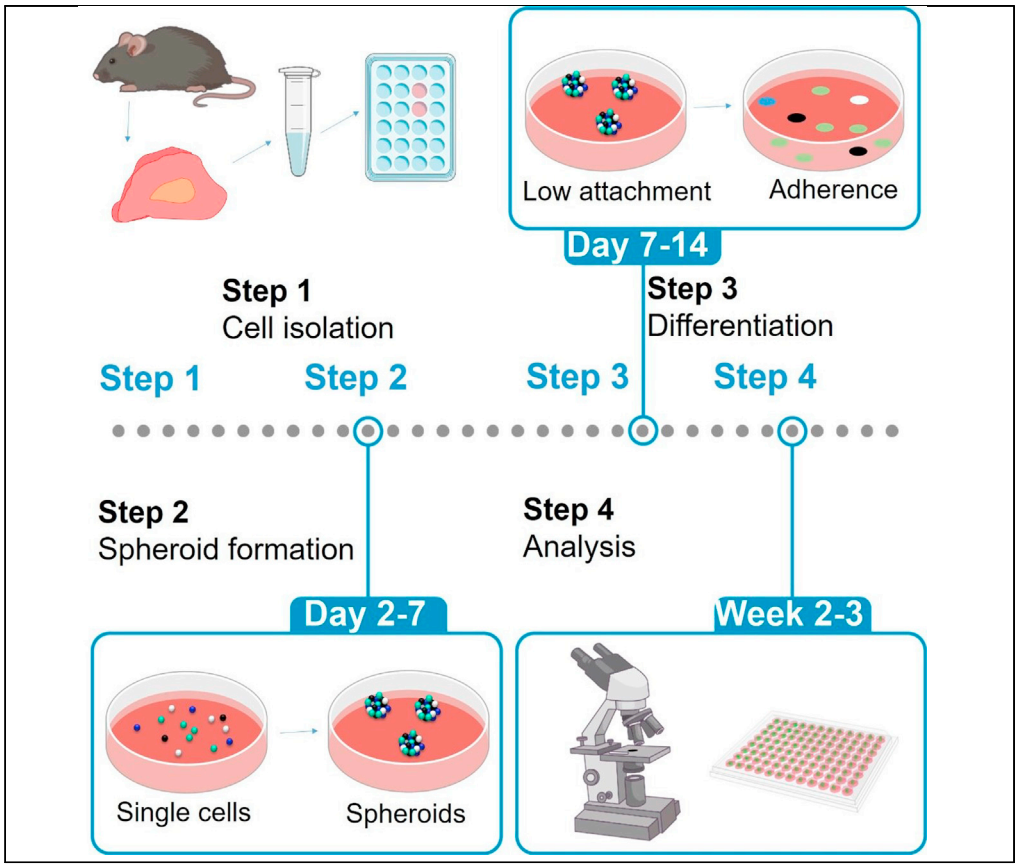
Isolation and *in vitro* cultivation of adrenal cells from mice



The adrenal gland consists of two tissues, cortex and medulla, united under one capsule. Adrenal stem/progenitor cells play a key role in development and homeostasis. Here, we describe a protocol for generating primary cultures of adrenal cells from mice. We describe techniques for separating the cortex and medulla, generating spheroid cultures containing stem- and progenitor cells, and for the differentiation into steroidogenic and chromaffin cells, respectively. This protocol enables analysis of various treatments before, during, or after differentiation.

Linda Friedrich, Maria Schuster, Maria F. Rubin de Celis, Ilona Berger, Stefan R. Bornstein, Charlotte Steenblock

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Highlights

Protocol for establishment of adrenal spheroids from mice

Adrenal spheroids contain cells possessing stem cell characteristics

Differentiation into steroid-producing cells or into chromaffin cells

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Isolation and *in vitro* cultivation of adrenal cells from mice

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SUMMARY

The adrenal gland consists of two tissues, cortex and medulla, united under one capsule. Adrenal stem/progenitor cells play a key role in development and homeostasis. Here, we describe a protocol for generating primary cultures of adrenal cells from mice. We describe techniques for separating the cortex and medulla, generating spheroid cultures containing stem- and progenitor cells, and for the differentiation into steroidogenic and chromaffin cells, respectively. This protocol enables analysis of various treatments before, during, or after differentiation.

For complete details on the use and execution of this protocol, please refer to Rubin de Celis et al. (2015), Steenblock et al. (2018), and Werdermann et al. (2021).

BEFORE YOU BEGIN

This protocol is focused on the isolation and differentiation of adrenocortical and adrenomedullary stem cells and progenitors from Nestin-GFP mice. However, the protocol can also be used for isolation of other adrenal cells and from other mouse strains, where the cells are not fluorescently labelled. The protocol provides different experimental conditions and final assessments that might not all be required by the experimental design pertinent to the addressed question. Please refer to the flow-chart (Figure 1) to define which experimental design will be suitable for your specific question. For example, if you have no interest in stem- and progenitor cells, it is possible to culture isolated and fully differentiated cells by skipping step 3 (proliferation) (Figure 1).

Before you start the isolation of adrenal cells, be aware that you need to have permission to perform animal experiments. In our case, all animal experiments were approved by the Regional Council of Saxony (Landesdirektion Sachsen) according to German animal welfare regulations (Licence TVA 17/2017).

We normally isolate adrenal cells from around 10 mice from both sexes per experiment, where all cells are pooled after separation into cortex and medulla, respectively. However, it is also possible to use the protocol for less or more mice.

General preparations

Timing: 30–60 min

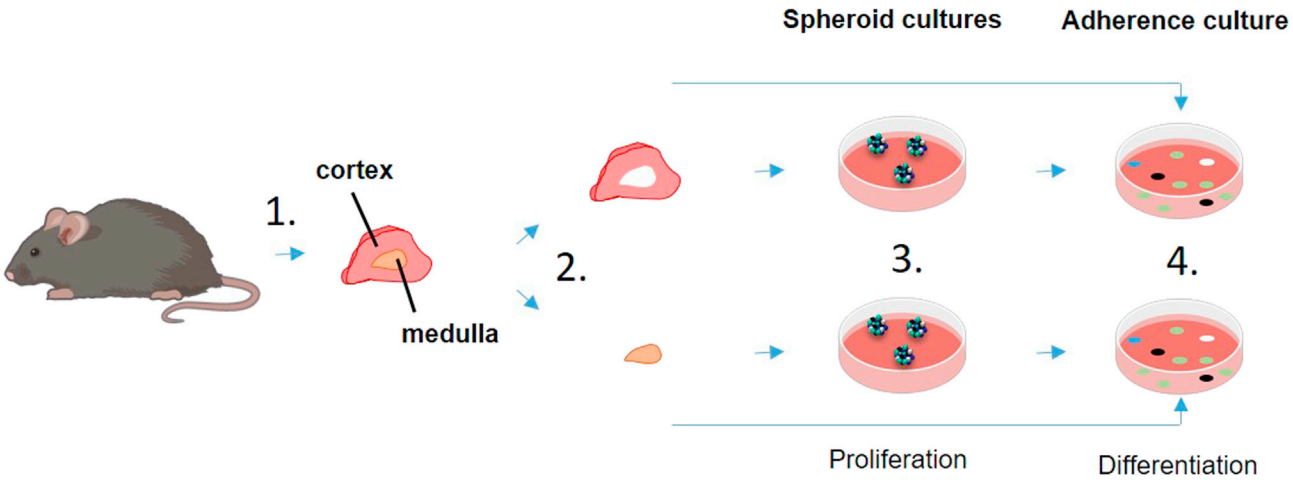

Figure 1. Flow chart with the experimental setup

The chart shows the full protocol, which however can be used for individual purposes: (1) isolation of adrenals, (2) separation of the adrenal into cortex and medulla, (3) spheroid culture of stem-like cells, (4) differentiation into mature adrenal cells.

1. Prepare all stock solutions
2. Prepare media
3. Sterilize all tools (scissors, forcipes) for dissecting the mice and collecting adrenals
4. All procedures are performed in a Class II biological hood with standard aseptic technique and primary cells are cultured in a humidified 37°C incubator with 5% CO_2.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Ethanol 80 V/V	Berkel AHK	Cat#7505F
PBS	Merck	Cat#D8537
DMEM/F12 medium	Gibco	Cat#31330-038
FBS Superior	Merck	Cat#S0615
Antibiotic-Antimycotic	Gibco	Cat#15240-096
L-glutamine	Merck	Cat#G7513
bFGF (hBFGF)	Merck	Cat#F0291-25 UG
BSA (Albumin Fraktion V)	Carl Roth	Cat#8076.4
Collagenase from *Clostridium histolyticum*	Merck	Cat#C6885
DNase I	Merck	Cat#D5025-150 kU
Poly-D-lysine	Merck	Cat#A-003-E
Fibronectin	R&D Systems	Cat#1030-FN
Experimental models: Organisms/strains		
Mouse: Nes-GFP, ~8 weeks old, both sexes	Mignone et al. (2004)	N/A
Software and algorithms		
ImageJ	Fiji	Version 1.52h
ZEN	Carl Zeiss MicroImaging GmbH	Version 2.3
Other		
Micro dissection forceps (straight)	F.S.T.	Cat#11254-20
Micro dissection forceps (bent)	Fine Science Tools	Cat#11274-20
Fine scissors	Fine Science Tools	Cat#14041-10

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Petri dish 60 × 15 mm	Greiner Bio-One	Cat#628161
Centrifuge	Thermo Scientific	Multifuge X1R
Stereomicroscope	Carl Zeiss	Stemi 2000; KI1500 LCD
Water bath	JULABO	SW23
Steriflip-GF 0.22 μm	Merck	Cat#SCGP00525
Easy strainer 100 μm	Greiner Bio-One	Cat#542000
Corning Costar ultra-low attachment 24 well plate	Corning	Cat#3473
24 well plate	Corning	Cat#353047
μ-slide 8 well uncoated	ibidi	Cat#80821

Note: General laboratory consumables such as serological pipettes (5 mL, 10 mL, 25 mL, 50 mL), p10, p20, p200 and p1000 pipettes and pipette tips are also required. The 24-well ultra-low attachment plates (https://ecatalog.corning.com/life-sciences/b2b/DE/en/Microplates/Assay-Microplates/96-Well-Microplates/Costar%C2%AE-Multiple-Well-Cell-Culture-Plates/p/3473?pagePath=p/3473) are critical for the protocol whereas other reagents can also be ordered from other suppliers.

MATERIALS AND EQUIPMENT

Preparation of stock solutions		
Reagent	Stock concentration	Amount
DNAse I	5 mg/mL in PBS	5 mL
bFGF	25 μg/mL in PBS	1 mL

Note: Both solutions can be prepared and stored for at least 2 years at −20°C until use.

Preparation of digestion buffer		
Reagent	Final concentration	Amount
Collagenase	1.8 mg/mL	18 mg
BSA	10 mg/mL	100 mg
DNase	0.18 mg/mL	360 μL (from 5 mg/mL stock)
PBS	–	Up to 10 mL

Note: First dissolve BSA and Collagenase lyophilized powder in PBS at 37°C. Add DNase and filter through a 0.22 μm filter (Steriflip-GP; Merck). Should be prepared fresh and used immediately. This amount of digestion buffer (10 mL) is sufficient for up to 20 adrenals, where 5 mL is used for digestion of the cortices and 5 mL is used for digestion of the medullae.

Preparation of culture medium		
Reagent	Final concentration	Amount
L-Glutamine	2 mM	500 μL (from 200 mM stock)
Antibiotic-Antimycotic	1×	500 μL (from 100× stock)
FBS Superior	10% (V/V)	5 mL
DMEM/F12	–	Up to 50 mL
bFGF	0.025 μg/mL	Add freshly to the volume needed (from 25 μg/mL stock)

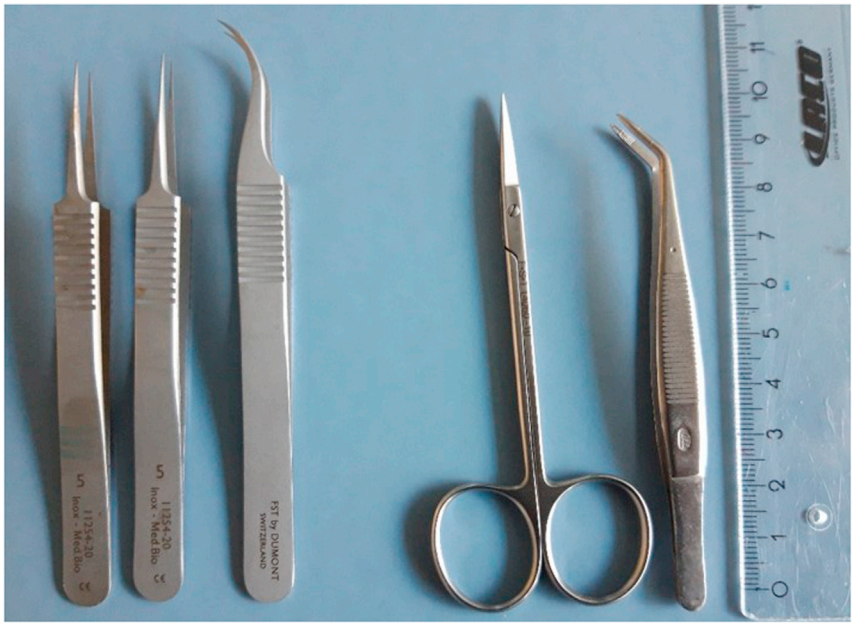

Figure 2. Tools for dissection

Note: The medium containing FBS, L-Glutamine and Antibiotic-Antimycotic can be stored at 4°C for up to 1 week. bFGF should be added fresh and the final solution should be used immediately. Since all reagents are cell culture tested and sterile, it is not necessary to filter them. The DMEM/F12 medium already contains L-Glutamine but because of its instability and the long-term culture, additional L-Glutamine should be supplied to the culture medium. To avoid different growth patterns with changing FBS batches, we use FBS superior. In this serum, growth-promoting compounds are supplemented to get a more consistent FBS. Thereby, we never experienced culture differences. We have also tested serum-free alternatives such as neurobasal medium (Gibco) supplemented with 2% B27 (Gibco). However, here the cells grow much slower and the morphology is changed. Both media contain high concentrations of glucose (17.5–25 mM) and we have not tested whether low glucose concentrations (<6 mM) influence the growth.

STEP-BY-STEP METHOD DETAILS

Preparations before start

Timing: 20–30 min

1. Prepare four small petri dishes (60 × 15 mm) filled with PBS, place them on ice

Note: Often more petri dishes are required during the procedure, especially when a larger number of mice are dissected and the PBS gets unclear. Therefore, it might be an advantage to prepare 1–2 additional dishes with PBS at the beginning.

2. Prepare tools for dissection (see Figure 2: forceps and scissors)
3. Prepare digestion buffer and preheat to 37°C
4. Prepare the culture medium and preheat it to 37°C

Note: For one experiment, 20 mL of culture medium is usually adequate. For long-term cultures, fresh culture medium will have to be prepared once per week. bFGF should always be added just before use.

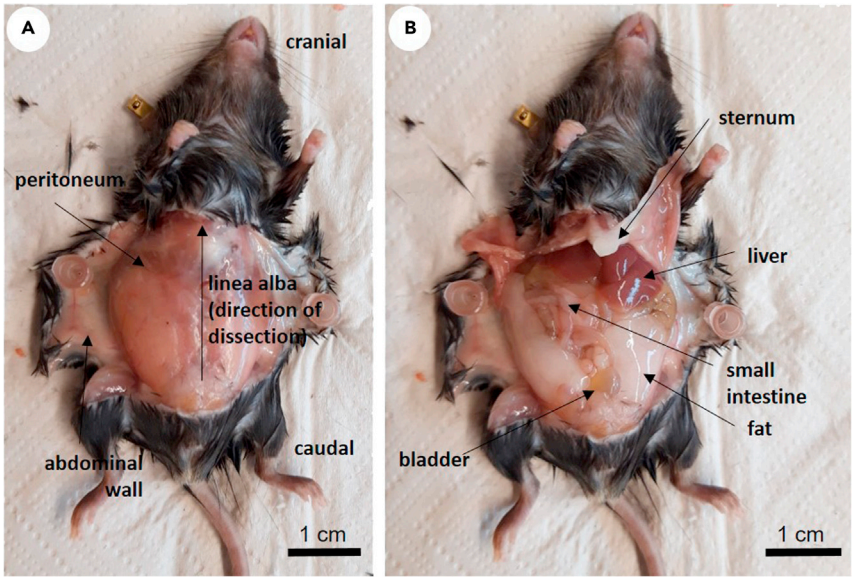

Figure 3. Mouse dissection
(A) Mouse after opening the abdominal wall. Scale bar, 1 cm.
(B) Mouse after opening the peritoneum. Scale bar, 1 cm.

⚠ CRITICAL: Sterility is crucial; the entire hood and materials should be clean. Troubleshooting 3.

Collecting adrenals

Timing: ~30 min

In this step, mice will be dissected and adrenals will be collected.

Note: We usually isolate adrenal cells from 10 mice from both sexes at an age of approximately 8 weeks. However, adrenal cells can be isolated from mice of all ages though the dissection of the adrenal glands gets very difficult when the mice are younger than 3 weeks. Similar protocols have been published previously for isolation of rat and murine adrenal cells. These protocols do not contain procedures for spheroid cultures and subsequent differentiation, however, they contain videos that might improve the visualization of the separation into cortex and medulla (Chen and Huang, 2019; Kolski-Andreaco et al., 2007).

5. Sacrifice the mouse according to the regional animal welfare act or/and the animal experiment application.
6. To avoid contamination spray 80% ethanol on the fur of the mouse (abdomen).
7. Open the abdominal wall by cutting in the middle (linea alba) from the bottom to the top (cranial) until the beginning of the diaphragm (Figure 3A).
8. Make a window by cutting at the top (cranial) and bottom (caudal) end of the previous cut both right and left.
9. Open the peritoneum in the same way as the abdominal wall before (Figure 3B).
10. To dissect the right adrenal gland, put all the organs (intestine, liver) very carefully to the left with forcipes (Figure 4A).

⚠ CRITICAL: Avoid dissection of other organs and vessels. If bleeding, it might be very difficult to find the adrenal. One possibility is then to flush with PBS.

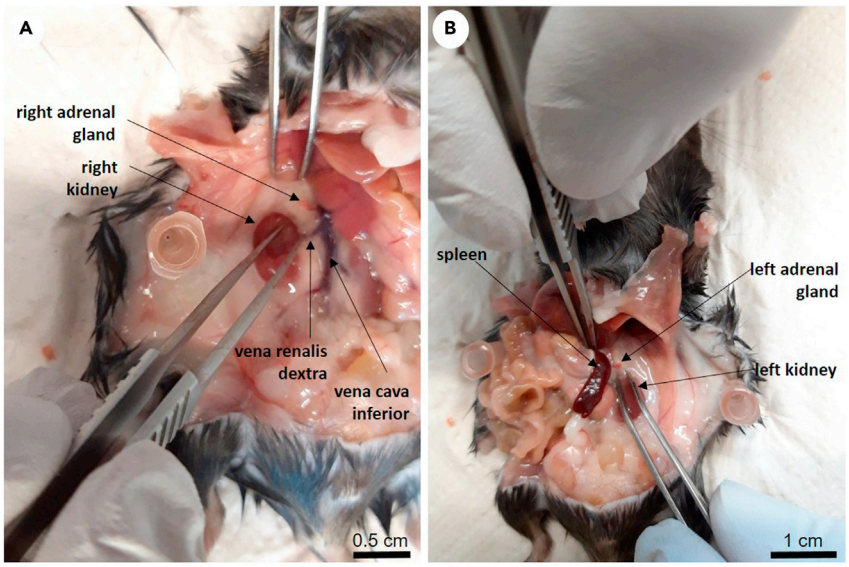

Figure 4. Adrenal location
(A) Right adrenal gland.
(B) Left adrenal gland.

11. Carefully lift up the left part of the liver to make the right kidney and adrenal gland visible.
12. Dissect the adrenal by grabbing some fat around the gland with a curved forceps without squeezing the gland itself.

⚠ CRITICAL: If the mouse has a lot of fat (Figure 3B) be careful not to dissect a vein or/and arteria since the vessels might be not visible.

13. Place the adrenal gland in a petri dish containing PBS on ice.
14. To dissect the left adrenal gland, put all organs very carefully to the right with forcipes (Figure 4B).
15. Carefully lift up the stomach and the spleen to make the left kidney and adrenal gland visible.
16. Dissect the adrenal by grabbing some fat around the gland with a curved forceps without squeezing the gland itself.
17. Place the adrenal in the petri dish with PBS already containing the right adrenal.
18. Repeat the dissection with the remaining mice and pool all adrenals in one petri dish.
19. To get rid of potential impurities and blood, carefully transfer all adrenals to a new petri dish with PBS on ice using a curved forceps.

Fat removal and separation into cortical and medullary tissue

Timing: 1–2 h

Note: The timing depends on the number of adrenal glands. After some practice, fat can be removed in 1–2 min per adrenal. The same is the case for the manual separation into cortex and medulla. However, the first couple of times, it might take much longer.

The following steps should be performed using a stereomicroscope. Troubleshooting 1.

20. Remove the fat surrounding the adrenal gland very carefully with two forcipes. The fat can be peeled off and discarded on a paper towel next to the petri dish (Figure 5A).

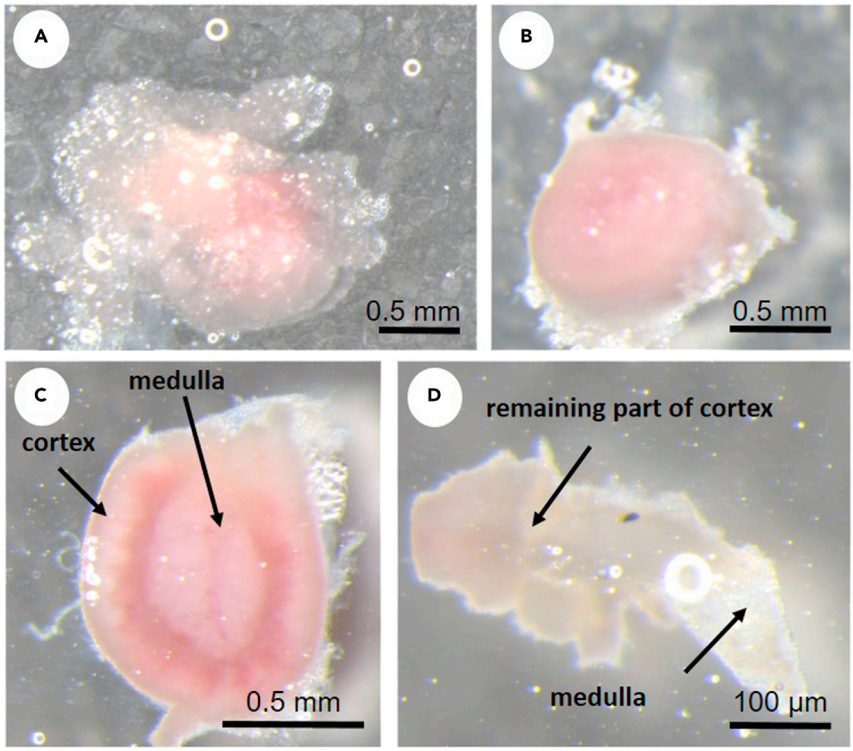

Figure 5. Fat removal and separation of cortex and medulla

(A) Adrenal gland with fat. Scale bar, 0.5 mm.

(B) Fat removed from adrenal. Scale bar, 0.5 mm.

(C) Opened adrenal gland. Scale bar, 0.5 mm.

(D) Medulla separated from cortex. Scale bar, 100 µm.

Note: Some prefer to use scalpels instead of forcipes. However, in our experience, you have better control while using forceps.

21. Put the cleaned adrenal glands into a new petri dish filled with PBS on ice. Continue with the remaining adrenal glands and collect them all in the same petri dish (Figure 5B).

⚠ CRITICAL: Do not compress and damage the adrenal gland while holding it with the forceps.

22. Place the petri dish with all cleaned adrenal glands under the stereomicroscope. Open one side of the adrenal gland and try to peel off the cortex little by little. The medulla will be visible but the PBS is getting unclear the more cortex is peeled off (Figure 5C).

Note: If the PBS gets too unclear, the remaining adrenal glands can be transferred to a new petri dish with PBS and then pooled again at a later point.

23. Use the curved forceps and carefully transfer the medulla into a new petri dish filled with PBS (Figure 5D). The cortical tissue pieces remain in the petri dish under the stereomicroscope. Continue with the remaining adrenal glands.

⚠ CRITICAL: As the medulla is very small, it is important to keep an eye on it the whole time. Otherwise, it might get difficult to distinguish it from the cortical tissue pieces.

Digestion and cell separation

Timing: ~60 min

24. Transfer the content of the petri dishes (cortex and medulla, respectively) to one 50 mL tube each. Use a 1000 μL pipette.

Note: The tissue might stick on the inside of the pipette tip. Try to pipette up and down to get out the remaining tissue.

25. Centrifuge for 5 min at 270 × *g* at room temperature (20°C–25°C).
26. Remove and discard the PBS supernatant using a 1000 μL pipette.
27. Add 5 mL digestion buffer, prepared as described in the "materials" section, to the 50 mL tubes containing the cortex and the medulla, respectively.
28. Place for 20 min in a shaking water bath at 37°C.

⚠ CRITICAL: The medullary cells are more sensitive than the cortical cells. Therefore, it is very important not to digest these cells for more than 20 min. For the cortical cells, 30 min will also be ok. Troubleshooting 2.

29. Take out the 50 mL tubes and resuspend the remaining tissue parts by pipetting up and down ~50 times using a 1000 μL pipette.
30. Centrifuge for 5 min at 270 × *g* at room temperature.
31. Washing step:
    a. Remove and discard the supernatant from both 50 mL tubes.
    b. Add 5 mL PBS to each 50 mL tube.
    c. Centrifuge for 5 min at 270 × *g* at room temperature.
32. Medullary cells:
    a. Remove and discard supernatant.
    b. Resuspend the cell pellet in 500 μL culture medium with bFGF.
    c. Add the suspension to an Easy strainer 100 μm pore size.
    d. Add another 500 μL to the strainer to get all cells through.
33. Cortical cells:
    a. 2nd washing step: Remove and discard the supernatant.
    b. Add 5 mL PBS to each 50 mL tube.
    c. Centrifuge for 5 min at 270 × *g* at room temperature.
    d. Remove and discard supernatant.
    e. Resuspend the cell pellet in 500 μL culture medium with bFGF.
    f. Add the suspension to an Easy strainer 100 μm pore size.
    g. Add another 500 μL to the strainer to get all cells through.
34. Seed each cell suspension in one well of an ultra-low attachment surface 24-well plate.
35. Culture the cells 16–24 h in the incubator (37°C, 5% CO_2).
36. Change medium by transferring the cells to a 15 mL tube using a 1000 μL pipette and centrifuge the cells at 270 × *g* for 5 min, remove old medium and resuspend the cells in fresh cell culture medium containing bFGF. Transfer cells to a new well of the 24-well plate.

Note: Depending on the initial amount of mice/adrenals that was pooled, it may be necessary to separate the cortical cells into 2–3 wells at the first medium change. In our experiments, we mostly use 10 mice and here we isolate enough cortical cells for 3 wells on a 24-well plate. Medullary cells are kept together in one well.

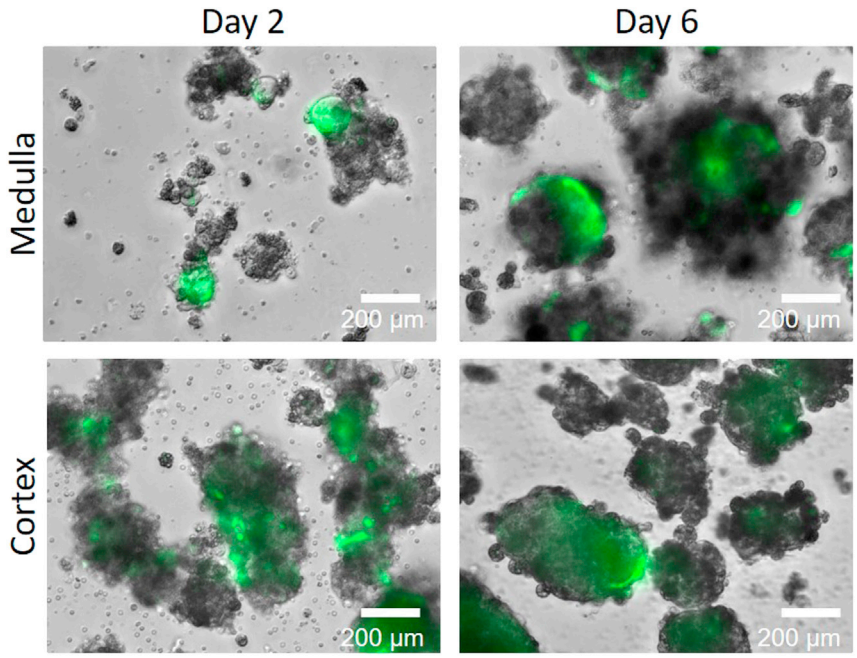

Figure 6. Proliferation and spheroid formation

1–2 days after seeding of dissociated cells (from 10 mice) in 24-well low-attachment plates, cells start to aggregate. After 3–4 days, spheroids are created. Nestin-GFP labelled cells are shown in green. Scale bars, 200 µm.

37. Culture cells for up to 4 weeks in spheroid cultures (Figure 6) by performing medium change every 3–4 days by carefully removing half of the medium and adding the same amount of new culture medium (in total 1 mL/well) containing bFGF.

Note: During this time, different stimulations can be added.

Differentiation protocol

Timing: 7–28 days

In case cells are going to be differentiated, culture conditions have to be changed to adherence and bFGF has to be omitted from the medium.

38. Coat a 24-well plate or an 8-well chamber slide with poly-D-lysine 16–24 h (300 µL/well, 1 mg/mL poly-D-lysine in PBS) in the incubator at 37°C.

Optional: For imaging, 8-well chamber slides from e.g. ibidi give the best results. Otherwise, (for collecting medium and/or RNA) normal 24 well plates can be used for differentiation.

39. Wash wells 3 times carefully with PBS
40. Add Fibronectin (300 µL/well, 0.6 nM in PBS) and incubate for 3 h at 37°C in the incubator.
41. Wash 3 times carefully with PBS
42. Transfer spheroids to the coated 8-well chamber slides:
    a. Carefully transfer spheroids to an 1.5 mL tube using a 1000 µL pipette.
    b. Centrifuge for 5 min at 270 × *g*.
    c. Add 1 mL new culture medium without bFGF.
    d. Distribute spheroids to all chambers and fill up with medium (300 µL/well)

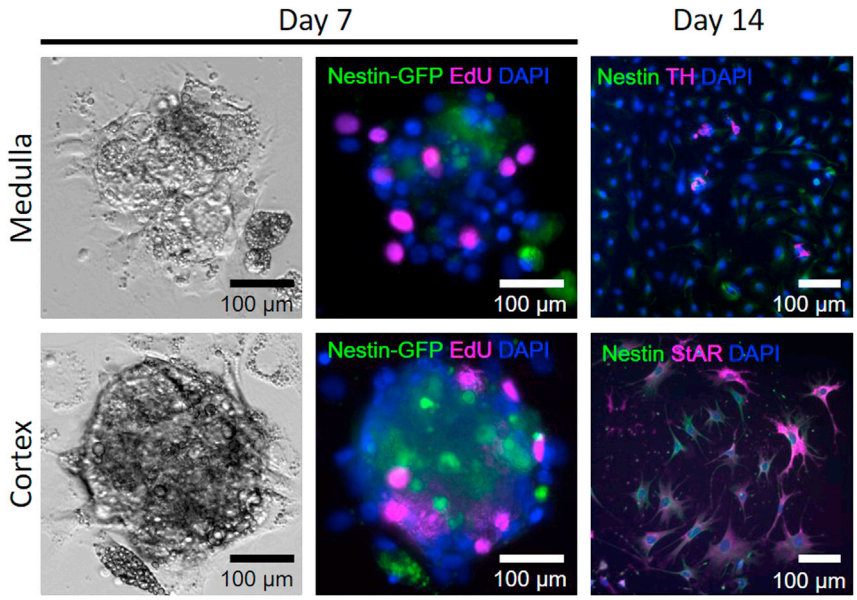

Figure 7. Adrenal cell differentiation

After at least 6 days of proliferation, cells are transferred to adherence conditions, where they will start to differentiate. 3 h after transfer, cells have started to adhere and proliferating cells can be identified by EdU-staining. After 7 days of differentiation (day 14), cells have spread out from the spheroids. Furthermore, most cells have lost their expression of Nestin and become positive for differentiation markers such as StAR (cortex) and tyrosine hydroxylase (medulla). Scale bars, 100 µm.

43. After 3–4 h cells have attached and will start differentiating
44. Medium should be changed 2 times per week by removing old medium and adding new. Then cells can be cultured for at least 28 days.

EXPECTED OUTCOMES

This protocol is for isolation and culture of adrenal cells from both cortex and medulla. Using this protocol, you can test your treatment of interest either during the proliferation (Figure 6) or differentiation process (Figure 7) or when cells are fully differentiated (Figure 7).

Amount of cells expected: From one C57BL/6 mouse (2 adrenals), 50–100.000 cells are normally isolated from the medulla, and 500.000–1.000.000 cells are isolated from the cortex. These numbers depend on the age of the mouse and the sex, as adrenals from female mice are bigger than those from males (Bechmann et al., 2021a). The protocol has not been tested with other mouse strains.

The proliferation of the cultured cells can be examined during both the proliferation and the differentiation phase using EdU staining as described by the manufacturer (https://www.thermofisher.com/order/catalog/product/C10340). The expression of different stem- and progenitor cell markers, such as *Nestin*, *Gli1*, and *Shh*, in the spheroids can be investigated using quantitative RT-PCR. For primer details, please see Steenblock et al. (2018). After 1 week in culture, the spheroids reach their final size with an average of ~100 µm in diameter.

The isolated and cultivated cells from the adrenal cortex show steroidogenic properties. The expression of steroidogenic markers, e.g., StAR, SF-1, CYP11A1, or CYP11B2, can be examined at the RNA level by quantitative RT-PCR or at the protein level by immunofluorescence staining. For details of antibodies and primers, please refer to (Steenblock et al., 2018). Furthermore, the secretion of

steroids into the cell culture medium can be measured using ELISA or mass spectrometry (Steenblock et al., 2018; Werdermann et al., 2021).

The isolated and cultivated cells from the adrenal medulla differentiate into chromaffin cells, glial cells, and neurons. The expression of stem-/ progenitor markers such as Nestin, or markers for more differentiated cell phenotypes such as Chromogranin A, Tyrosine hydroxylase, and β-tubulin can be examined by isolating RNA for quantitative RT-PCR or at the protein level by immunofluorescence staining. For details of antibodies and primers, please refer to Rubin de Celis et al. (2015) and Steenblock et al. (2018). Catecholamine production in the cells can be measured using mass spectrometry as described previously (Bechmann et al., 2021b). In our experience, the medullary cells divide and grow faster than the cortical cells (Figure 7).

Critical commercial assays

REAGENT	SOURCE	IDENTIFIER
Click-iT EdU Cell Proliferation Kit for Imaging, Alexa Fluor 647 dye	Thermo Fisher Scientific	Cat#C10340
Aldosterone ELISA	IBL International	Cat#RE52301
Corticosterone ELISA	Demeditec	Cat#DEV9922

LIMITATIONS

The amount of cells achieved per adrenal and their ability to generate spheroids depends on the age of the mice. In our experience, the diameter of the spheroids is smaller when adrenal cells are isolated from older mice. Therefore, we normally use 8-week old mice and definitely mice younger than 8 months.

The cortex and the medulla are manually separated under the microscope. Here, it is impossible to reach 100% purity, and especially for the medulla, there will always be some adrenocortical cells attached to the medulla. However, as most stem- and progenitor cells in the adrenal cortex are located directly under the adrenal capsule and in the outer zona Glomerulosa, this usually does not influence the results.

TROUBLESHOOTING

Problem 1

Cortex and medulla are not separated properly (steps 22–23).

Potential solution

It takes some training to peel the cortex and separate the medulla properly. If the adrenal is cut, take your eyes on it and try not to damage the medulla while peeling off the cortex.

Problem 2

Many cells have died during the procedure (steps 24–34).

Potential solution

It is important to work as fast as possible to avoid that the cells spend too much time in just PBS. Furthermore, for the adrenal medulla it is very important that the dissociation into single cells is stopped after 20 min in the digestion buffer. The cortical cells are more robust and can stay for up to 30 min in the digestion buffer without dying. Furthermore, in order to increase the viability, it is important to always place the cells on ice whenever possible.

Problem 3

Bacterial or fungal contamination (step 37).

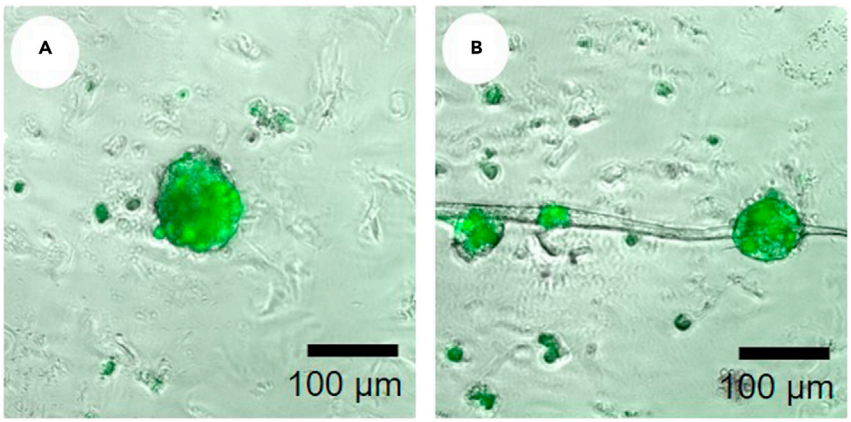

Figure 8. Impurities in the culture medium

(A) Successful spheroid formation with a diameter of ~100 µm. Scale bar, 100 µm.

(B) Cells aggregate at impurities in the medium (e.g., mouse hair) and several smaller spheroids are generated. Scale bar, 100 µm.

Potential solution

Spray 80% ethanol on the fur of the mouse before opening the abdomen. Use sterile instruments and sterile PBS. Clean the instruments in between with 80% ethanol. Start working under the laminar flow as soon as the cortex and the medullary cells are separated.

Problem 4

Impurities in the medium may give rise to aggregations of cells at these impurities (Figure 8; step 37).

Potential solution

Cells and solutions are filtered but sometimes small impurities such as mouse hairs end up in the culture plates anyway. Make sure that you work as clean as possible.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Charlotte Steenblock (charlotte.steenblock@ukdd.de).

Materials availability

This study did not generate unique reagents. All reagents are available as specified in the key resource table.

Data and code availability

This study did not generate/analyze [datasets/code].

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

M.F.R established the initial protocol; L.F. and M.S. performed research, made images, and prepared the first draft of the manuscript; I.B. and C.S. performed research and optimized the protocol. C.S. wrote the final version of the paper; S.R.B. and C.S. supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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