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Isolation and Culture of Primary Neurons and Glia from Adult Rat Urinary Bladder

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Corresponding Author:	Hong-ying Cao Guangzhou University of Chinese Medicine Guangzhou, CHINA
Corresponding Author's Institution:	Guangzhou University of Chinese Medicine
Corresponding Author E-Mail:	hycao@gzucm.edu.cn
Order of Authors:	Rui Wang
	Hong-ying Cao
	Zi-tong Huang
	Wen-kang Ren
	Jiao Zhang
	Yao Zhang
	Bo Tan
	Ping Huang
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TITLE:

Isolation and Culture of Primary Neurons and Glia from Adult Rat Urinary Bladder

AUTHORS AND AFFILIATIONS:

Rui Wang[1], Zi-tong Huang[3], Wen-kang Ren[1], Jiao Zhang[1], Yao Zhang[1], Bo Tan[3], Ping Huang[1,2], Hong-ying Cao[1,2]

[1]School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine, Guangzhou, China
[2]Dongguan & Guangzhou University of Chinese Medicine Cooperative Academy of Mathematical Engineering for Chinese Medicine, Guangzhou University of Chinese Medicine, Dongguan, China
[3]School of Basic Medical Sciences, Guangzhou University of Chinese Medicine, Guangzhou, China

Emails of Authors
Rui Wang
wangrui@stu.gzucm.edu.cn

Zi-tong Huang
823805602@qq.com

Wen-kang Ren
510894139@qq.com

Jiao Zhang
2291728353@qq.com

Yao Zhang
1036809630@qq.com

Bo Tan
tannyhy@gzucm.edu.cn

Ping Huang
hping331@126.com

Hong-ying Cao
hycao@gzucm.edu.cn

Corresponding Author:
Hong-ying Cao
hycao@gzucm.edu.cn

KEYWORDS:

Neuroscience, primary neurons, primary glia, isolation, culture, neuron subtypes

SUMMARY:
This protocol attempts to establish a repeatable protocol for primary neurons and glia isolation from rat bladder for further cellular experiments.

ABSTRACT:
The lower urinary tract has two main functions, namely, periodic urine storage and micturition; these functions are mediated through central and peripheral neuroregulation. Although extensive research on the lower urinary tract nervous system has been conducted, most studies have focused on primary culture. This protocol introduces a method for the isolation and culture of bladder neurons and glia from Sprague–Dawley rats. In this method, the neurons and glia were incubated in a 37 °C, 5% CO_2 incubator for 5–7 days. As a result, they grew into mature shapes suitable for related subsequent immunofluorescence experiments. Cells were morphologically observed using an optical microscope. Neurons, synaptic vesicles, and glia were identified by β-III-tubulin and MAP-2, Synapsin-1, and GFAP staining, respectively. Meanwhile, immunocytochemistry was performed on several neurotransmitter-related proteins, such as choline acetyltransferase, DYNLL2, and SLC17A9.

INTRODUCTION:
The lower urinary tract has two main functions: periodic urine storage and micturition[1]. The lower urinary tract nervous system (LUTNS) controls these functions and is delicate and susceptible to many neuropathies, which can be innate (porphyria), acquired (Lyme disease), secondary to disease states (diabetic cystopathy), drug induced (hemorrhagic cystitis), surgery caused (abdominoperineal resection), or injury caused (traumatic spinal cord injury)[2-7]. In physiological/pathological studies, in vivo and in vitro experiments are equally important. While in vivo research on LUTNS has been conducted at organ, cellular, and molecular levels for some time, in vitro research on primary neurons from the urinary bladder is almost nonexistent[8,9]. Although the present study is limited, we hope to pioneer research in this area so that other researchers could improve it. In this manner, this co-culture may lead to a cellular understanding of physiological dysfunction in phenotypes, such as bladder neuron dysfunction.

In contrast to enteric muscles with a clear directionality of the muscle cells into discrete layers, the muscles of the bladder are unorganized[10]. Therefore, instead of peeling off the outer layer of the bladder, this method proposes digesting the entire bladder to reduce the difficulty of operation and shorten the preprocessing time for a high cell survival rate.

Following this method, we can obtain a mixed culture of neurons and other cells. The other cells are indispensable because their presence mimics an in vivo environment[11]. In addition, such cells provide the substances that are unavailable in the medium.

This method involves two steps for digestion. First, collagenase type II is used to hydrolyze collagen, followed by trypsin, to dissociate the tissue into cells[10]. In this manner, bladder tissues are dispersed into single cells and then grow relatively independent. When the culture of neurons matures, the neurons can be used for imaging or functional assays.

PROTOCOL:

All experimental protocols and animal procedures complied with the ethical principle guidelines of the National Research Council.

1. Preparation of materials

1.1. Sterilize all instruments and ddH_2O using an autoclave before performing the experiment. Instruments include but are not limited to surgical scissors, ophthalmic scissors, forceps, spoons nucleus divider, glass dishes (60–100 mm in diameter), and glass breakers.

1.2. Prepare the Krebs solution as follows (**Table 1**): Dissolve all the chemicals together with ddH_2O prior to use. Dissolve $CCaCl_2$ separately and add slowly into the mixed solution while stirring to avoid sediment.

1.3. Prepare the Rinse media, which consists of F12 media with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (100x). Add 5 mL of FBS and 0.5 mL of antibiotic/antimycotic to 44.5 mL of F12 media.

1.4. Prepare neuron media A, which comprises neurobasal A media with 2% B-27, 1% FBS, 1% L-glutamine, 1% antibiotic/antimycotic (100x), and 0.01% glia-derived neurotrophic factor (GDNF). Add 200 µL of B-27, 100 µL of FBS, 100 µL of L-glutamine, 100 µL of antibiotic/antimycotic (100x), and 10 µL of GDNF (10 µg/mL) to 9.5 mL of neurobasal A media.

NOTE: Prepare neuron media A within one week of usage to ensure freshness of B-27, L-glutamine and GDNF.

1.5. Prepare neuron media B using the same procedure as the preparation of neuron media A but without adding FBS.

1.6. Prepare digestion solution 1 by dissolving 20 mg of collagenase type II, 6 mg of bovine serum albumin, and 200 µL of antibiotic/antimycotic (100x) in 10 mL of oxygen-stable Krebs solution.

1.7. Prepare digestion solution 2 by diluting 1 mL of 0.25% trypsin with 4 mL of Hank's balanced salt solution.

1.8. Prepare a plate with coated coverslips.

1.8.1. Use forceps in a laminar flow bench when placing glass coverslips into a 48-well culture plate.

1.8.2. Dilute poly-D-lysine with ddH_2O to a concentration of 0.1 mg/mL as poly-D-lysine stock. Store the stock at –20 °C, and thaw before use.

1.8.3. Add 40 µL of poly-D-lysine stock on top of each coverslip, and incubate the solution at room temperature for 10 min.

NOTE: For different plates with different basal areas, adjust the concentration to 4 µg/cm^2. For example, if the basal area of one well from a 24-well plate is 2 cm^2, then we should transfer 80 µL of poly-D-lysine stock in a well. Each cm^2 would contain 4 µg of poly-D-lysine.

1.8.4. Remove poly-D-lysine in the 48-well plate, and rinse coverslips with ddH_2O thrice.

1.8.5. Dry the plate in a laminar flow hood for at least 30 min to ensure that the plate is anhydrous.

1.8.6. Store the plate at 4 °C before laminin coating for 1 day at most. Storing the plate at −20 °C is preferable for long-term storage.

1.8.7. Thaw laminin at 4 °C. Dilute laminin with ddH_2O to a concentration of 50 µg/mL as laminin stock. Store the stock at –20 °C, and thaw at 4 °C before use.

1.8.8. Use a pipette to transfer 100 µL of diluted laminin to the top of each coverslip and incubate laminin at 4 °C for 1 h.

NOTE: For different plates with different basal areas, adjust the concentration to 5 µg/cm^2. For example, if the basal area of one well from a 24-well plate is 2 cm^2, then transfer 200 µL of diluted laminin in a well. Each cm^2 would contain 5 µg of laminin.

1.8.9. Remove the laminin solution, and rinse coverslips with ddH_2O once. Perform this operation on the edge of the coverslip to avoid scraping.

NOTE: Coated coverslips could be stored at 4 °C for 2 weeks at most.

2. Bladder harvest

2.1. Obtain five-week-old Sprague–Dawley rats.

2.2. Infuse carbogen (95% oxygen, 5% CO_2) into the Krebs solution for at least 30 min in an ice bath to reach a stable oxygen status and pH level.

2.3. After euthanasia via cervical dislocation, soak the rats in 75% ethanol for 30 s for sterilization.

2.4. Place rats on a sterilized surgical towel and expose their abdomen. Open the abdominal cavity, and reveal the bladder with a set of scissors and forceps.

2.5. Lift the bladder gently and cut the bladder from the bladder neck with another set of scissors

and forceps to avoid cross-contamination. Place the bladder rapidly in cold oxygen-stable Krebs solution to improve cell survival.

NOTE: Once the bladder is removed, perform the following operations rapidly to improve the prospect of neuron survival.

2.6. Add Krebs solution into three glass dishes and glass breakers.

2.7. Prepare glass dishes and glass breakers with Krebs solution in an ice bath for precooling.

2.8. Mark these containers with numbers 1–3 correspondingly to prevent confusion.

2.9. Pair each glass dish with forceps and a spoons nucleus divider.

2.10. In glass dish 1, cut open the bladder with ophthalmic scissors, and unfold it with forceps and a spoons nucleus divider.

2.11. Rinse the bladder in glass breaker 1, and place it in glass dish 2.

2.12. Eliminate adherent fat on the tissue surface using the forceps and ophthalmic scissors in glass dish 2.

2.13. Rinse the bladder in glass breaker 2, and place it in glass dish 3.

2.14. Gently scrape the bladder using the forceps and the spoons nucleus divider onto glass dish 3 to remove exogenous attachments.

2.15. Rinse the bladder in glass breaker 3, transfer the bladder to a 15 mL centrifuge tube with 14 mL of cold Krebs solution, and spin the sample for 1 min at 356 x *g* and 4 °C.

2.16. Repeat the previous step twice in two other tubes with Krebs solution to reduce contamination.

3. Two-step bladder digestion

3.1. Transfer the bladder from the centrifuge tube to a 2 mL vial containing 1 mL of digestion solution 1. Use ophthalmic scissors to cut the bladder into small pieces (smaller than 1 mm) in solution.

3.2. Mix the bladder solution with 9 mL of digestion solution 1 in a sterile cell culture dish (100 mm in diameter). Perform step 1 digestion in a shaking incubator for 1 h under 5% CO_2, 37 °C, and 200 rpm.

3.3. After step 1 digestion, centrifuge the solution at 356 x *g* at 4 °C for 8 min.

3.4. Place digestion solution 2 in a 37 °C water bath for preheating.

3.5. After centrifugation, remove the supernatant that contains digestion solution 1, and harvest the cell sediment. Some remaining liquid is allowed. Complete removal of the solution may promote cell loss.

3.6. Mix cell sediment with warm digestion solution 2 in a 15 mL centrifuge tube, and shake the mixture while digesting in a 37 °C water bath for 5 min. Do not exceed 7 min of step 2 digestion or neurons will perish.

3.7. After digestion, immediately deactivate trypsin in the mixture with 10 mL of cold rinse media.

NOTE: Perform the following steps at 0 °C–4 °C. An ice bath could provide such condition.

3.8. Harvest the cell sediment after centrifugation at 356 x *g* at 4 °C for 8 min. Remove as much media as possible because the remaining trypsin is harmful to cell growth.

3.9. Resuspend sediment with 3 mL of neuron media gently. Ensure that air bubbles are not generated in the solution containing cells for a high survival rate.

3.10. Filter the mixture media through a 70 µm cell strainer into a 50 mL centrifuge tube.

3.11. Keep the filtrate on a shaker at 30 rpm in an ice bath for 30 min. This step is not necessary but recommended.

3.12. Collect cells by centrifugation at 356 x *g* at 4 °C for 8 min, and gently resuspend the cell pellets in 1 mL of neuron media A.

3.13. Add 500 µL of cell mixture into each well of the prepared 48-well plate.

3.14. Culture cells in an incubator at 37 °C and 5% CO_2.

3.15. Replace all the media with neuron media B in 1 h to provide a serum-free culture.

3.16. Change half of the neuron media B every 3 days.

NOTE: Neurons are ready for immunocytochemical experiments after 5–7 days of culture.

REPRESENTATIVE RESULTS:

In the process of primary cell culture, the cells acquired were round with bright and clear boundaries before the attached state. As the neurons grew, dendrites and axons started to be distinct. After 5–7 days of culture, the neurons reached a mature form with long projections, which were ideal for imaging or function studies. Although most of the impurities and cell debris

could be removed due to changing media, certain residuals attached to poly-D-lysine and laminin coating were visible (**Figure 1**).

After proper culture, neurons could be identified via typical β-III-tubulin and MAP-2 immunostaining[10,12]. In addition, glia was specifically identified via GFAP immunostaining[10]. Mature neurons developed synaptic spines, which were close to the presynaptic specializations identified by the immunostaining of the synaptic protein maker, synapsin-1 (**Figure 2**)[12]. These results indicated that mature cells with well-developed synapses were obtained through this method. This result suggests its important role in future function studies.

Meanwhile, several neuron subtypes were recognized through immunocytochemistry experiments (**Figure 3**). Peptidergic neurons, which contain various neuropeptides, were immunostained with substance P[13]. Purinergic neurons with expressed vesicular nucleotide transporters were identified via SLC17A9 staining[14]. Nitrergic neurons were visualized with DYNLL-2, which connects nNOS with motor proteins in neurons[15]. Cholinergic neurons were immunoreactive with choline acetyltransferase[16].

FIGURE AND TABLE LEGENDS:

Figure 1. Phase-contrast images of primary cells isolated from the rat bladder culture taken at 1, 3, and 7 days after plating (A, B, C, respectively). Scale bar: 50 μm.

Figure 2. Immunofluorescence images of primary cells isolated from the rat bladder. Confocal microscopy analysis showed neuron cytoskeleton protein staining (β-III-tubulin, RRID: AB_2827688, 1:200) in primary culture neurons (**A**) and in whole mount bladder preparation (**B**). In primary culture neurons, neuronal phosphoprotein immunostaining (MAP 2, RRID:AB_2827689, 1:200) was also visualized (**C**). Glia were identified via glial fibrillary acidic protein staining (**D**; RRID: AB_627673, 1:50). Synapsins were visualized via synapsin protein staining (Synapsin-1, RRID: AB_2798146, 1:200) in cellular (**E**) and tissue (**F**) levels. The secondary antibodies used were as follows: Alexa Fluor 488 (green, goat anti-rabbit lgG, 1:200), Alexa Fluor 555 (red, goat anti-mouse lgG, 1:200). The nucleus was visualized using Hoechst 33342 (**A, C, D, E**; blue, 1 μg/mL). Scale bar: 50 μm.

Figure 3. Immunofluorescence images of several neuron subtypes of primary neurons. Peptidergic neurons were immunostained with substance P (**A**; RRID: AB_785913, 1:50). Purinergic neurons were identified via SLC17A9 staining (**B**; RRID: AB_10597575, 1:200). Nitrergic neurons were visualized via DYNLL-2 staining (**C**; RRID: AB_654147, 1:50). Cholinergic neurons were immunoreactive with choline acetyltransferase (**D**; RRID: AB_2244867, 1:100). The secondary antibodies used were as follows: Alexa Fluor 488 (green, goat anti-rabbit lgG, 1:200), Alexa Fluor 555 (red, goat anti-mouse lgG, 1:200). The nucleus was visualized using Hoechst 33342 (**A, B, C, D**, blue, 1 μg/mL). Scale bar: 50 μm.

Table 1. Krebs solution composition

DISCUSSION:

Plate Preparation

The use of glass coverslips in 6-, 12-, or 48-well culture plates for immunofluorescent or calcium imaging experiments is an economical and sample-sparing operation. Cells grow well in plates without coverslips during the preparation of primary cell cultures. Therefore, coverslips are dispensable in experiments, such as Western blot or polymerase chain reaction. Furthermore, coating is a necessary step before plating cells, with or without coverslips. Laminin and poly-D-lysine are common choices in coating neurons, particularly laminin, which is essential for neuron growth[17].

Media Preparation

After first medium replacement, cell isolation requires media without serum because serum stimulates cell division and leads to a limited neuron-growing space[18]. Thus, neuron growth factors are crucial. The quality of B27 and GDNF can vary largely from different batches and cause major effects on neuron growth[19]. Therefore, checking the lot number of the media is recommended when neuron yield is poor. Meanwhile, fresh media stock is crucial; the required amount should be calculated and prepared in advance each time before media are replaced.

Animals

Sprague–Dawley rats are used in this method. C57BL/6 mice are also acceptable in this experiment. Therefore, other strains of rats or mice may also be adopted for this method despite a few variations in morphology and neuronal circuitry. In terms of different animal models, researchers should develop an optimized and targeted protocol. Furthermore, young animals should always be considered prior to the application of this method.

Tissue Treatment

During the experiments, except for the digestive process, keeping tissues at a low temperature is essential to increase cell viability, which can reduce cell metabolism and avoid energy deficit. Oxygen levels, nutrition, and pH can also affect cell yield[11]. Moreover, for other tissues, we suggest that researchers perform this method with adjusted digestion condition.

Cell Culture

One remarkable characteristic of neurons when inoculated is their quick adherence to coated plates[20]. In this case, changing media after 1 h of culture is recommended to gain a high proportion of neurons. Moreover, when most of the cells start to grow pseudopodium, the frequency of changing media can be reduced appropriately depending on the color of the media and the cellular state. Primary cell culture in a good state displays black soma with a bright border.

Most nerve cells isolated from the bladder are bladder intramural ganglia, which consist of afferent and autonomic efferent innervations of the bladder[13]. Moreover, no major pelvic ganglia are present in the harvested tissue. It distributes below the bladder neck[21].

Limitation

This is a preliminary research to isolate and culture neurons and glia. Many attempts had been

done, like cytarabine treatment or density gradient centrifugation. However, the proportion of desired cells was still not ideal, and even more cell loss appeared. Moreover, traditional digestive conditions in this protocol, such as 37 °C, are likely to kill off some sensitive neuron types, and cause potential gene expression artifacts[22].

In conclusion, this protocol offers a method to culture neurons and glia from rat bladder. The isolation is easy to repeat, time efficient, and involves minimal microbial contamination. Although improvement evoking the purity of neurons is necessary, we hope this method contributes to LUTNS research.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare no major conflict of interest.

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Figure 1

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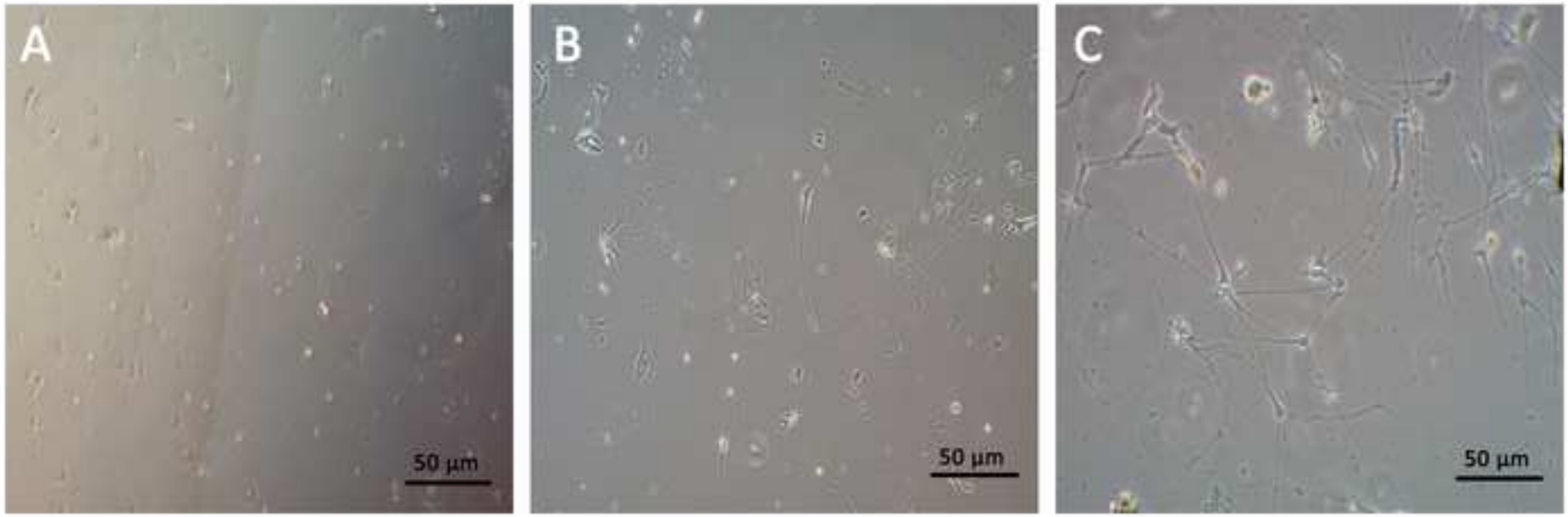

Figure 2

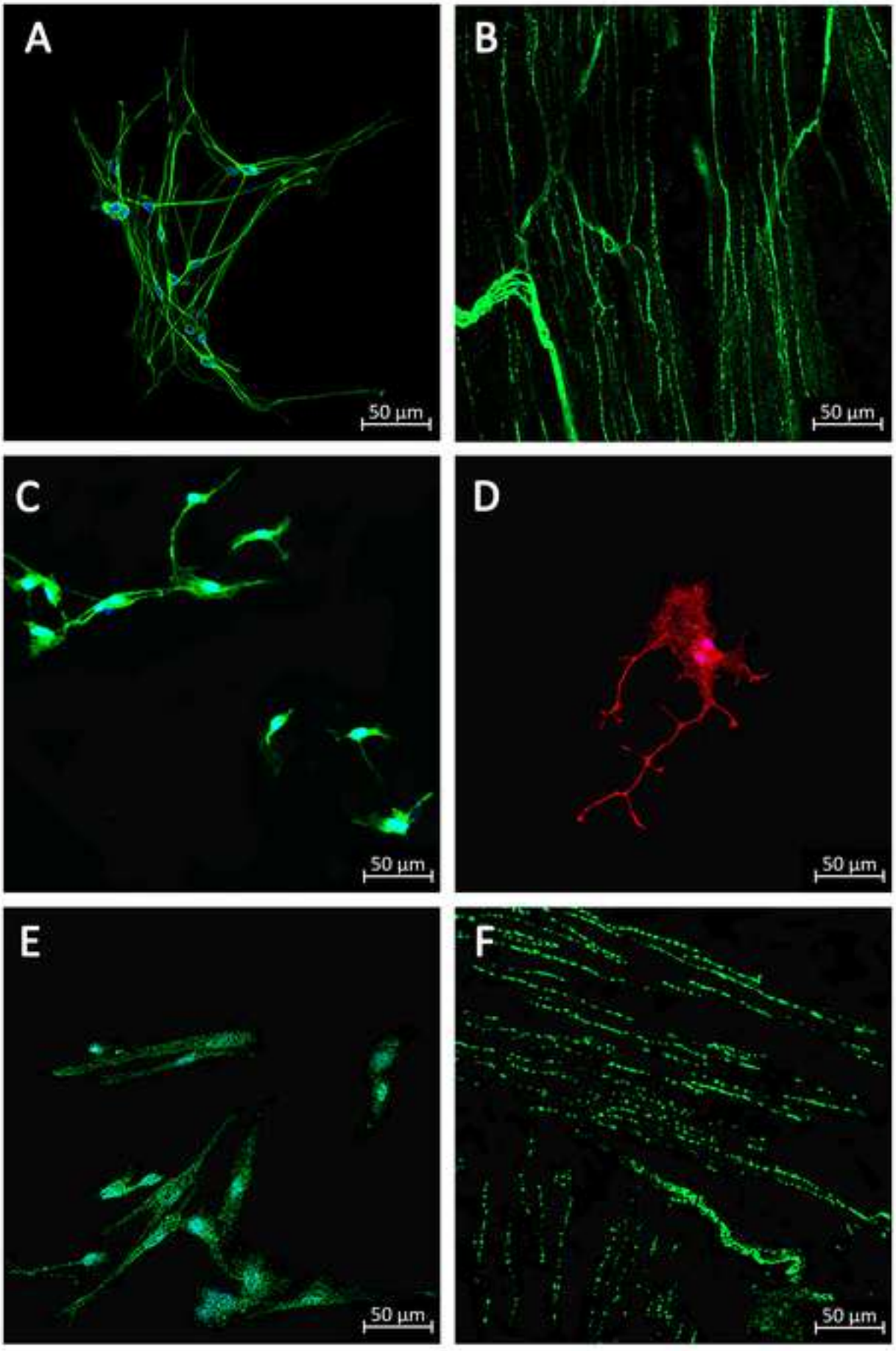

Figure 3

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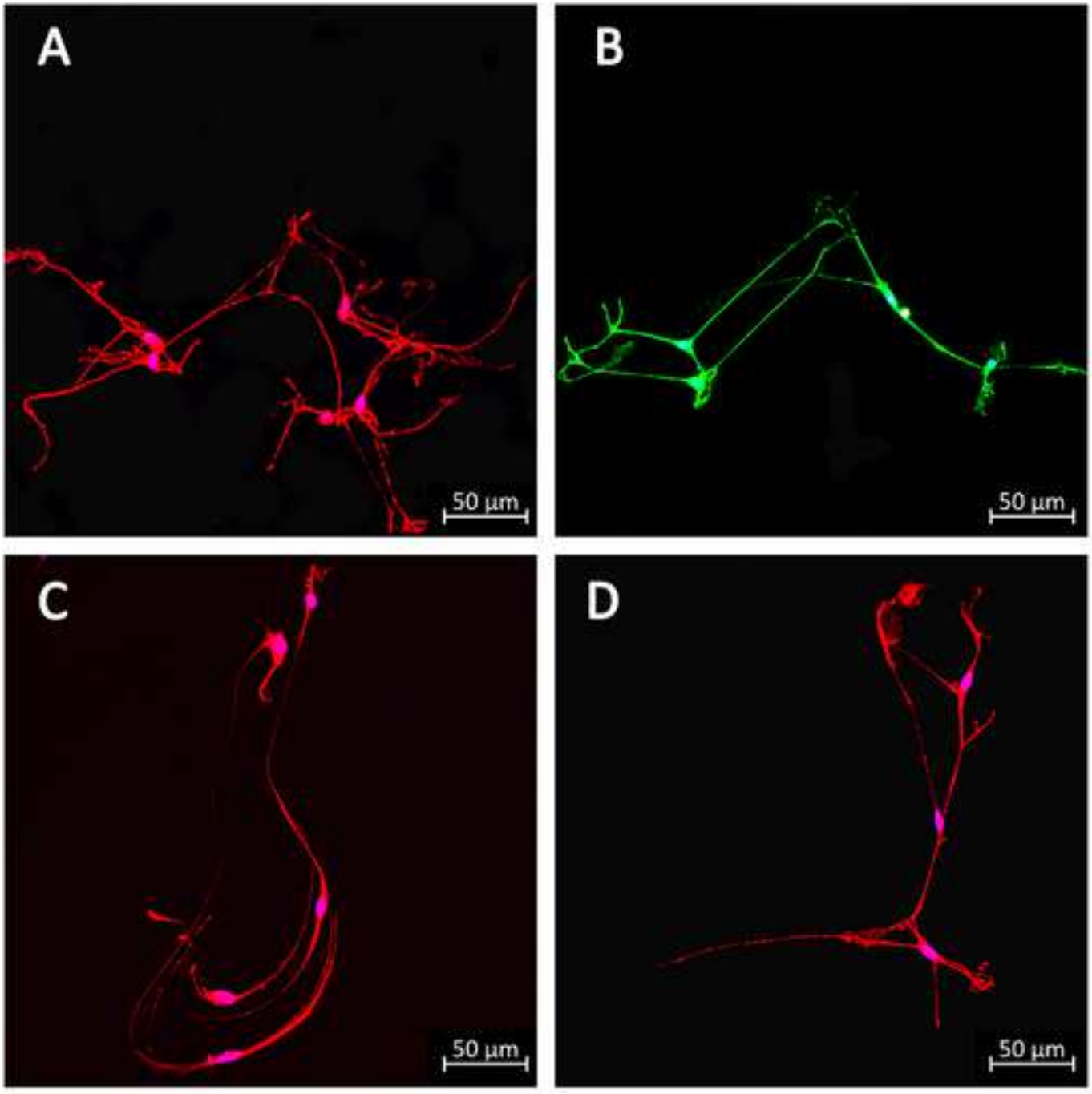

Table 1

Ingredients	Molarity (mM)
NaCl	120
KCl	5.9
$NaHCO_3$	25
$Na_2HPO_4 \cdot 12H_2O$	1.2
$MgCl_2 \cdot 6H_2O$	1.2
$CaCl_2$	2.5
Glucose	11.5

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.25% trypsin	Gibico	15050065	Enzyme digestion
48-well culture plate	Corning	3548	Coating dish
antibiotic/antimycotic	Gibico	15240062	Culture media/Rinse media
Anti-Glial Fibrillary Acidic Protein Antibody	Santa Cruz	sc-33673	ICC
B-27	Gibico	17504044	Culture media
BSA Fraction V	Gibico	332	Enzyme digestion
Choline Acetyltransferase Antibody	Abcam	ab18736	ICC
CO_2 Incubator	Heraeus	B16UU	Cells culture
Collagenase type II	Sigma	2593923	Enzyme digestion
DMEM/F-12	Gibico	11330032	Rinse media
DYNLL2 Antibody	Santa Cruz	sc-13969	ICC
Fetal Bovine Serum	Gibico	10100147	Culture media/Rinse media
Forceps	Shanghai Jin Zhong Medical Devices	1383	10 cm; Sterile operation
Glass breakers	Huan Qiu Medical Devices	1101	50 ml; Sterile operation
Glass coverslips	WHB Scientific	WHB-48-CS	Coating dish
Glass dishes	Huan Qiu Medical Devices	1177	100 mm; Sterile operation
Goat Anti-Rat IgG(H+L), Mouse ads-Alexa Fluor 488	Southernbiotech	3050-30	ICC
Goat Anti-Rat IgG(H+L), Mouse ads-Alexa Fluor 555	Southernbiotech	3050-30	ICC
Hoechst 33342	BD	561908	ICC
Laminar flow bench	Su Jie Medical Devices	CB 1400V	Sterile operation
Laminin	Sigma	L2020	Coating dish
L-glutamine	Gibico	25030081	Culture media
MAP-2 Antibody	Affinity	AF5156	ICC
Murine GDNF	Peprotech	AF45044	Culture media
Neurobasal-A Medium	Gibico	10888022	Culture media
Ophthalmic scissors	Shanghai Jin Zhong Medical Devices	J21010	12.5 cm; Sterile operation
Pipettes	Eppendorf	3120000240	100-1000 ul; Reagent and sample pipetting
Pipettes	Eppendorf	3120000267	10-100 ul; Reagent and sample pipetting
Poly-D-lysine	Sigma	P7280	Coating dish
Refrigerated centrifuge	Ping Fan Instrument	TGL-16A	Enzyme digestion
Shaking incubator	Haimen Kylin-Bell Lab Instruments	T8-1	Enzyme digestion
SLC17A9 Antibody	MBL International	BMP079	ICC
Spoons nucleus divider	Shanghai Jin Zhong Medical Devices	YZR030	12 cm; Sterile operation
Substance P Antibody	Santa Cruz	sc-58591	ICC
Surgical scissors	Shanghai Jin Zhong Medical Devices	J21130	16 cm; Sterile operation
Surgical towel	Fu Kang Medical Devices	5002	40 x 50 cm; Sterile operation
Synapsin-1 Antibody	CST	5297T	ICC
Tubulin beta Antibody(β-III-tubulin)	Affinity	AF7011	ICC

Editorial comments:

1. Summary: "This protocol attempts to offer…" is unclear; please make this more succinct.

Our response: Thank you for your comments. Summary was changed into "This protocol attempts to establish a repeatable protocol for primary neurons and glia isolation from rat bladder for further cellular experiments" in the revised manuscript.

2. Please clarify if 2.6-2.8 and 2.16 should be highlighted for filming.

Our response: Thank you for your reminding. 2.6-2.8 should be highlighted and 2.16 should not be highlighted for filming. We have modified these in the revised manuscript. The highlight with yellow is the protocol ready for filmable content, while the highlight with red is the corrections to identify all of the edits.

3. Please remove the embedded table from the manuscript and instead upload as an Excel file.

Our response: Thank you for your comments. We have removed the embedded table and uploaded a corresponding Excel file.

4. Please cite Figure 3 outside of the Figure legends section.

Our response: Thank you for your reminding. We have cited Figure 3 in REPRESENTATIVE RESULTS part of the revised manuscript.

5. Figure 1: Is panel A 1 day, B 3 days, and C 7 days after plating?

Our response: Thank you for your reminding. Yes, it is. We have clarified those in FIGURE AND TABLE LEGENDS part of the revised manuscript.

6. Figure 2, 3: The scale bars are still hard to distinguish against the background; please use a color that contrasts better.

Our response: Thank you for your reminding. We have used white to contrast better in figure 2, 3.

They authors have addressed all my concerns.

Our response: Thank you for your approval.

Manuscript Summary:
The present work aims to explain how we can obtain primary neurons and glia from the bladder of adult rats. Although every step is described in detail, there are some major and minor concerns about this work.

Major Concerns:
My major concern about this protocol is the fact that authors are culturing neurons from the bladder itself. Most of the primary afferents (if not all) are in the dorsal root ganglia. The autonomic nervous system has extramural ganglia. In fact, one of the reasons why botox doesn't work or has low efficacy when applied in bladder of rats is that the cell body of neurons (in general) are outside the bladder wall. This is supported by figure 2B, that only shows nerve fibres. Furthermore, Beta-3-tubulin (here used as a neuronal markers) has been shown to be expressed by normal mesenchymal and epithelial cells (among others... see Jouhilahti et al, at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2583904). Hence, it is difficult to me to understand what neurons are being plated in this work. Can authors explain?

Our response: Thank you for your comments. Researchers declared that detrusor muscles in fetal rats and mice were densely innervated, possessing abundant intramural ganglia[1,2]. And with the development of urinary system, there are a small number of intramural ganglia preserving in the bladder of adult rats and mice, which leads to difficulty in increasing the proportion of primary neurons[2,3]. Furthermore, extirpation of the pelvic ganglion could induce intramural neurons appearing in the urinary bladder wall, suggesting plasticity of bladder innervation[4,5]. Therefore, plated neurons might be from intramural ganglia and neuronal differentiation[6]. Moreover, except for Beta-3-tubulin, MAP 2 was also used to identify neurons in figure 2C, which is widely used as a neuronal marker[7,8].

[1]. Danzer, Enrico et al. “Structural and functional characterization of bladder smooth muscle in fetal rats with retinoic acid-induced myelomeningocele.” American journal of physiology. Renal physiology vol. 292,1 (2007): F197-206.

[2]. Georgas, Kylie M et al. “An illustrated anatomical ontology of the developing mouse lower urogenital tract.” Development (Cambridge, England) vol. 142,10 (2015): 1893-908.

[3]. McNeill, D L et al. “Origin and distribution of NADPH-diaphorase-positive neurons and fibers innervating the urinary bladder of the rat.” Neuroscience letters vol. 147,1 (1992): 33-6.

[4]. Uvelius, B, and G Gabella. “Intramural neurones appear in the urinary bladder wall following excision of the pelvic ganglion in the rat.” Neuroreport vol. 6,16 (1995): 2213-6.

[5]. Uvelius, B, and G Gabella. “The distribution of intramural nerves in urinary bladder after partial denervation in the female rat.” Urological research vol. 26,5 (1998): 291-7.

[6]. Wiese, Carrie B et al. “Migration pathways of sacral neural crest during development of lower urogenital tract innervation.” Developmental biology vol. 429,1 (2017): 356-369.

[7]. Pino, Darya et al. “Wnt5a controls neurite development in olfactory bulb interneurons.” ASN neuro vol. 3,3 e00059. 2 Jun. 2011.

[8]. Roppongi, Reiko T et al. “Low-Density Primary Hippocampal Neuron Culture.” Journal of visualized

experiments : JoVE ,122 55000. 18 Apr. 2017.

Minor Concerns:

It is not clear how authors change media in step 3.15 without losing the cells that, at that moment, are not attached yet.

Our response: Thank you for your comments. There exits slight cell loss. However, this step aims to increase the proportion of neurons, which have quick adherence to coated plates[1]. Meanwhile, laminin and poly-D-lysine play a role in promoting attachment of cells[2,3]. And after preliminary experiments, we chose to replace all the media in 1 h.

[1]. Kaech, Stefanie, and Gary Banker. "Culturing hippocampal neurons." Nature protocols vol. 1,5 (2006): 2406-15.

[2]. Hayashi, Hideki et al. "Biological activities of laminin-111-derived peptide-chitosan matrices in a primary culture of rat cortical neurons." Archives of biochemistry and biophysics vol. 648 (2018): 53-59.

[3]. Kim, Yong Hee et al. "Enhancement of neuronal cell adhesion by covalent binding of poly-D-lysine." Journal of neuroscience methods vol. 202,1 (2011): 38-44.

Also, how did authors prevent non-neuronal cell growth? After 5-7 days of culture, if not "blocked", non-neuronal cell will cover all the slides/dishes. However, when observing the figures, the cells seem to be quite isolated.

Our response: Thank you for your comments. To prevent non-neuronal cell growth, we use neurobasal A media with serum-free culturing and GDNF, B27 as supplement in reference to former studies. Serum stimulates cell division and leads to limited neuron-growing space[1]. Therefore, we replace all the media with serum-free media in 1 h when most neurons have attached. Neurobasal A media are widely used in neuron culture and allows for long-term maintenance of the normal phenotype and growth of neuronal cells[2]. And GDNF could significantly enhance dendritic arborization[3]. Moreover, B27 is an optimized serum-free supplement used to support the low- or high-density growth and short- or long-term viability of neurons[4].

[1]. Kivell, B M et al. "Serum-free culture of rat post-natal and fetal brainstem neurons." Brain research. Developmental brain research vol. 120,2 (2000): 199-210.

[2]. Gustafsson, Julie Ry et al. "Neurobasal media facilitates increased specificity of siRNA-mediated knockdown in primary cerebellar cultures." Journal of neuroscience methods vol. 274 (2016): 116-124.

[3]. Politi, L E et al. "Effect of GDNF on neuroblast proliferation and photoreceptor survival: additive protection with docosahexaenoic acid." Investigative ophthalmology & visual science vol. 42,12 (2001): 3008-15.

[4]. Majd, Shohreh et al. "Culturing adult rat hippocampal neurons with long-interval changing media." Iranian biomedical journal vol. 12,2 (2008): 101-7.