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TITLE:

Apoptosis Induction and Detection in a Primary Culture of Sea Cucumber Intestinal Cells

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KEYWORDS:

sea cucumber, primary culture, cell culture medium, intestinal cells, apoptosis, marine invertebrate cell culture

SUMMARY:

This protocol provides an easy-to-handle method to culture the intestinal cells from sea cucumber *Apostichopus japonicus* and is compatible with a variety of widely available tissue samples from marine organisms including Echinodermata, Mollusca, and Crustacea.

ABSTRACT:

Primary cultured cells are used in a variety of scientific disciplines as exceptionally important tools for the functional evaluation of biological substances or characterization of specific biological activities. However, due to the lack of universally applicable cell culture media and protocols, well described cell culture methods for marine organisms are still limited. Meanwhile, the commonly occurring microbial contamination and polytropic properties of marine invertebrate cells further impede the establishment of an effective cell culture strategy for marine invertebrates. Here, we describe an easy-to-handle method for culturing intestinal cells from sea cucumber *Apostichopus japonicus*; additionally, we provide an example of in vitro apoptosis induction and detection in primary cultured intestinal cells. Moreover, this experiment provides details about the appropriate culture medium and cell collection method. The described protocol is compatible with a variety of widely available tissue samples from marine organisms including Echinodermata, Mollusca, and Crustacea, and it can provide sufficient cells for multiple in vitro experimental applications. This

technique would enable researchers to efficiently manipulate primary cell cultures from marine invertebrates and to facilitate the functional evaluation of targeted biological materials on cells.

INTRODUCTION:

Culturing cells under artificially controlled conditions, and not in their natural environment, provides uniform experimental materials for biological studies, especially for species which cannot be easily cultured in a laboratory environment. Marine invertebrates account for more than 30% of all animal species[1], and they provide numerous biological materials for undertaking research on the regulatory mechanisms of specific biological processes, such as regeneration[2,3], stress response[4], and environmental adaptation[5,6].

The sea cucumber, *Apostichopus japonicus*, is one of the most studied echinoderm species inhabiting temperate waters along the North Pacific coast. It is well known as a commercially important species and maricultured on a large scale in East Asia, especially in China[7]. Numerous scientific questions regarding *A. japonicus*, including the regulatory mechanisms underlying intestinal regeneration after evisceration[8] and degeneration in aestivation[9], metabolic control[10,11], and immune response[12,13] under thermal or pathogenic stresses, have attracted the attention of researchers. However, compared with well-studied model animals, basic research, especially on the cellular level, is limited by technical bottlenecks, such as the lack of advanced cell culture methods.

Researchers have devoted much effort to establishing cell lines, but they have also faced many challenges and no cell line from any marine invertebrate has been established yet[14]. However, primary cell cultures from marine invertebrates have advanced in last decades[15,16], and they have provided an opportunity for experimentation on the cellular level. For example, the regenerating intesine from *A. japonicus* has been utilized as a source of cells for long-term cell cultures which provided a practical method for primary cell culture of marine invertebrates[17]. This protocol combined and optimized invertebrate cell culture approaches and developed a widely suitable primary culture method for sea cucumber or other marine invertebrates.

Apoptosis is an intrinsic cell suicide program triggered by various exogenous and endogenous stimuli. Coordinated apoptosis is crucial to many biological systems[18,19], and it has been implicated in the intestinal regression of sea cucumber during aestivation[9]. To investigate the apoptotic process in organisms of interest, a series of methods, including Hoechst staining and microscopy assays, have been established and successfully applied[20]. Here, we conducted apoptosis induction and detection in primary cultured intestinal cells of sea cucumber to assess the usability of primary cells in biological studies of marine invertebrates. Dexamethasone, one of the commonly used synthetic glucocorticosteroids[21], was used to induce apoptosis in cultured intestinal cells from sea cucumber, and significant

Hoechst 33258 signal was successfully detected in the stained cells by fluorescent microscopy.

PROTOCOL

1. Cell culture medium preparation

1.1. Coelomic fluid preparation

1.1.1. **Coelomic fluid collection**: Under sterile conditions, dissect a healthy sea cucumber (wet weight of 85-105 g), collect coelomic fluid, and store it in a sterile glass flask.

1.1.2. **Coelomic cell removal:** Centrifuge the coelomic fluid in 50 mL centrifuge tubes at 1,700 x *g* for 5 min and transfer the supernatant into a new sterile glass flask; next, collect the cell-free coelomic fluid of the sea cucumber.

1.1.3. **Complement components inactivation**: Incubate the sterile glass flask, containing the sea cucumber coelomic fluid, in a 40–50 °C water bath for 20–40 min to obtain complement components-inactivated coelomic fluid.

1.1.4. **Microbe removal**: Remove bacteria and chlamydia by filtration through 0.22 µm membrane filters. Next, remove mycoplasma and other fine particles by filtration using 0.1 µm membrane filters to obtain coelomic fluid pretreatment solution.

1.1.5. **Salinity adjustment**: Adjust the salinity of the coelomic fluid pretreatment solution to 30‰ (measured by salinometer) by adding 20% high concentration presterilized and filtered NaCl solution (diluted by pretreated coelomic fluid) or DDW (double distilled water). Transfer the sea cucumber coelomic fluid into a sterile bottle, seal the bottle, and store the fluid at 4 °C for further experiments.

1.2. Leibovitz's L-15 cell culture medium optimization

1.2.1. Weigh 5.05 g of NaCl, 0.135 g of KCl, 0.15 g of $CaCl_2$, 0.25 g of Na_2SO_4, 0.975 g of $MgCl_2$, 0.25 g of glucose, and 6.25 mg of taurine and dilute them in 40 mL of Leibovitz's L-15 medium in a 50 mL sterile centrifuge tube. Agitate the tube on a shaker for 1 h to ensure the salts have dissolved completely.

1.2.2. Add 2.5 mL of L-glutamine (100 mg/mL) and 500 µL of VE solution (1.75 mg/L) into previously prepared Leibovitz's L-15 medium and further filter the medium through 0.22 µm membrane filters.

1.2.3. Adjust the total medium volume to 500 mL with fresh Leibovitz's L-15 medium and with 100 mL of previously prepared coelomic fluid; next, adjust the pH to 7.6 using NaOH solution. Keep the operation process in a sterile environment. The compounding ratio of coelomic fluid can be 10%–50%, and 20% is sufficient for *A. japonicus* intestinal cells culture.

2. Intestinal cell preparation

2.1. Sea cucumber intestine processing

2.1.1. Anaesthetize healthy sea cucumbers in an ice box. Dissect and collect the anterior intestines, then section the tissue samples vertically and remove the inner contents.

2.1.2. Wash the tissue samples in phosphate buffered saline (PBS) twice and disinfect them by immersion in an aqueous ethanol solution (75% by volume) for no longer than 2 s.

2.1.3. Wash the tissue samples in PBS three times to remove ethanol and transfer about 100 mg of tissue sample into a 2.0 mL sterile microcentrifuge tube.

2.2. Cell collection

2.2.1. Add 1.5 mL of the pre-optimized culture medium to the sea cucumber intestinal tissue block and mince the block with sterilized surgical scissors until the solution is cloudy. For optional simplified protocol, add 0.5 mL of pre-optimized culture medium to the sea cucumber intestinal tissue block and cut the block with sterilized surgical scissors into 1 mm^3. Directly transfer the samples to culture dishes followed by subsequent incubating steps.

2.2.2. Add 400 µL of trypsin (0.25%), mix the solution by inversion, and incubate it for 5 min at room temperature; then, filter the solution using a 100 µm cell strainer.

NOTE: It is optional to add trypsin for cell dispersion when treating different tissue samples. Ethylenediaminetetraacetic acid (EDTA) should be contained in trypsin solution to reduce the inhibitory activity from Ca^{2+} and Mg^{2+} in culture medium.

2.2.3. Collect the filtrate to a new sterile 2.0 mL microcentrifuge tube, centrifuge at 1,700 x *g* for 3 min, discard the supernatant, then resuspend the pellet in culture medium (supplemented with antibiotics) and wash it twice.

NOTE: Prepare fresh pre-optimized culture medium supplemented with 2% penicillin-streptomycin solution (10,000 U/mL penicillin and 10 mg/mL streptomycin) and 1% gentamicin (4 mg/mL) before the beginning of the experiment.

3. Cell culture

3.1. Incubator presetting: Preset the incubator for cell culture and run it in advance for at least 24 h with temperature of 18 °C and saturated humidity. Feed CO_2 into the incubator depending on the cell culture medium properties; no CO_2 needs to be supplied when using the basic medium of Leibovitz's L-15.

3.2. First stage cell culture

3.2.1. To inhibit the growth of microbes and to promote the proliferation during the initial stage of cell culture, add 10 mL of penicillin-streptomycin solution (10,000 U/mL penicillin and 10 mg/mL streptomycin) and 0.5 mL of gentamicin (40 mg/mL) into every 500 mL of pre-optimized culture medium. Furthermore, supplement every 500 mL culture medium with 0.6 mL of insulin (10 mg/mL), 100 µL of insulin-like growth factor (0.1 µg/µL), and 25 µL of fibroblast growth factor (0.1 µg/µL).

3.2.2. Collect the cells into 1.5 mL tubes, resuspend them using 200 µL of indicated medium, and pipet them into ϕ 4 cm dishes.

3.2.3. Culture the cells in an incubator and add 2.0 mL of indicated medium to the cell culturing dishes after 6 h. Change half of the medium every 12 h until reaching the next stage.

NOTE: Handle the medium change gently, because the cells are not attached to the dishes tightly. Poly-D-lysine-coated dishes can be used for loosely attaching to the dish cells.

3.3. Second stage cell culture

3.3.1. To reduce the adverse effects of antibiotics to the cultured cells, reduce the concentration of indicated antibiotics (penicillin, streptomycin, and gentamicin) in the culture medium by half.

NOTE: The usage of insulin and growth factors depends on the cell culture conditions and is optional.

3.3.2. Replace the cell culture medium; conduct medium changes every two to three days depending on the cell density.

NOTE: Observe the cultured cells daily under a microscope and record the growth conditions.

3.4. Cell passaging

NOTE: Passage and subculture the cells, when the primary cell density reaches 60%.

3.4.1. Wash the cultured cells twice using PBS at room temperature. Add 200 µL of trypsin solution (0.25%) to each dish and manually agitate the dish ensuring the whole bottom is covered. Discard the trypsin solution and incubate the cells for 5 min at room temperature.

3.4.2. Wash the cells with 1.0 mL of fresh culture medium by pipetting and resuspending the cells. Transfer 0.5 mL of cell suspension to a new dish, add 1.5 mL of fresh medium, and incubate the cells at 18 °C.

NOTE: Cell scrapers can be used for cell collection when the trypsin solution fails to digest and detach cells from the dishes (some cell lines are too adhesive). However, do not conduct both methods simultaneously.

3.4.3. Change the medium after 12 h and observe the cells under a microscope to evaluate the conditions. Culture the cells for further experimental assays.

4. Apoptosis induction and detection in *A. japonicus* intestinal cells

4.1. Cell culture and dexamethasone treatment

4.1.1. Prepare intestinal cells following the previously introduced protocol and add the cells dropwise to a 12-well plate at a cell volume of 2×10^6 per well.

4.1.2. After three days in culture, following the steps of the “first stage cell culture”, wash the cells three times with PBS and replace the medium with optimized medium (without antibiotics and growth factors).

4.1.3. Dilute dexamethasone (DXMS) in culture medium to prepare fresh 2 µM and 200 µM DXMS solutions before beginning the experiments.

4.1.4. Add DXMS solutions in different concentrations to cultured cells grown with the same volume of culturing medium; set three experimental groups including control (CTL), 1 µM, and 100 µM DXMS.

4.2. Hoechst staining

4.2.1. Wash the cells with PBS three times after incubation with/without DXMS for the indicated time periods (0 h, 24 h, and 48 h).

4.2.2. Add 300 µL of Hoechst 33258 solution per well to a 12-well plate and incubate at 18 °C for 30 min. Gently agitate the plate to cover all cells ensuring their staining.

4.2.3. Remove the Hoechst staining solution and fix the cells by adding 300 µL of a 4% paraformaldehyde solution (in PBS) to each well. Gently agitate for 15 min.

CAUTION: Paraformaldehyde is moderately toxic by skin contact or inhalation, and it is designated as a probable human carcinogen. Chemical fume hoods, vented balance enclosures, or other protective measures should be used during the weighing and handling of paraformaldehyde.

4.2.4. Wash the fixed cells three times in PBS. Do not discard the PBS after washing to keep cells covered.

4.3. Fluorescent microscopy analysis

4.3.1. Turn on the fluorescent microscope hardware including the mercury lamp power, fluorescent light power, and PC. Log into the operating system account, launch the software, and check its configuration.

4.3.2. Place the prepared plate on the microscope stage. Position the sample over the objective lens using the stage controller.

4.3.3. Find the cells of interest under the light microscope, switch to fluorescent microscopy, and capture the images by tuning the parameters.

NOTE: To observe the Hoechst 33258 fluorescent signal bound to nuclei DNA, fluorescence microscopy should be conducted with excitation and emission at approximately 352 nm and 461 nm, respectively.

REPRESENTATIVE RESULTS

Here, we established primary intestinal cell culture of *A. japonicus* and passaged the cells. **Figure 1** shows round cells in different stages of culturing. And the EdU staining assays provide direct evidences to reveal the proliferative activity of these round cells in later stage (**Figure 2**). We also slightly adjusted the protocol, culturing minced tissue blocks instead of filtrated cells; furthermore, a spindle cell type could be cultured successfully. This cell type occurred around the intestinal tissue blocks and could be observed after four days of culture; however, it failed to be passaged (**Figure 3).**

The cultured cells can be used for different biological experimental applications. We treated the cells with DXMS for different time periods, followed by Hoechst 33258 staining for apoptotic cell detection. **Figure 4** indicates the induced apoptotic signal detected from sea cucumber intestinal cells by Hoechst 33258 staining and fluorescent microscopy. **Figure 5**

demonstrates the apoptotic cell rates for indicated time periods under stimulation with DXMS.

FIGURE LEGENDS

Figure 1. Microscopy of cultured sea cucumber intestinal round cells at different stages. (**A**) Cells attached to the bottom of dishes on the third day after being seeded. (**B**) Cell proliferation on the seventh day. (**C**) Cells attached to the bottom of dishes after passaging. (**D**) Cells which have lost activity and are dying after ten passages. “CM” indicates cell mass, which occurs during cell proliferation. “BT” indicates the black track, which was left by dead cells.

Figure 2. Cell proliferation detected by EdU staining assays. Cell proliferation assays were performed using the kit (**Table of Materials**) according to the manufacturer's protocol.

Figure 3. Microscopy of cultured sea cucumber intestinal tissue blocks and cell proliferation. (**A**) tissue blocks (TB) attached to the dish bottom on the first day after culture. (**B**) Tissue blocks and cells attached to the dish bottom on the second day after culture. (**C**) Tissue blocks and peripherally occurred spindle cells (SC) on the fourth day after culture. (**D**) proliferated spindle cells on the seventh day after culture.

Figure 4. Fluorescent microscopy of intestinal round cells with induced apoptosis. “NC” indicates nuclear condensation fluorescent signal.

Figure 5. Statistical analysis of Hoechst-positive intestinal round cells. Distribution of the percentage of nuclear condensation fluorescent signal-positive cells among Hoechst stained *A. japonicus* intestinal cells along with the dose or time course of the experiment (n = 3).

DISCUSSION:

Extensive research efforts have been devoted to establishing cell lines in last decades, however, it is still difficult to make a progress on long-term culture of cells from marine invertebrates[14,22]. It has been reported that cultured cells from regenerating holothurian tissues were viable for a long period of time and high activity of proliferation can be detected in specific cells[17,23]. However, for the normal marine invertebrate cells, there is yet no practical cell culture approach been described. The protocol presented here provides an efficient and easily interpretable method for culturing and passaging intestinal cells from the marine invertebrate species *A. japonicus*. The method can be easily adapted for primary cell culture of many different marine invertebrate organisms such as cuttlefish and crab.

A series of key factors affect the successful application of this procedure. The prevention of microbial contamination is of foremost importance during the whole process, and especially during the first week of culture. Aquatic animals usually contact numerous microbes from environment, which colonize their tissues, such as gill, intestine, fin, and skin. Thus, it will be

impossible to sterilize the tissue samples before the cell culture initiation. To minimize the risk of microbial contamination during the cell culture, 75% ethanol immersion for tissue sample sterilization is essential; however, the treatment time should be optimized for different tissue types. Moreover, the application of high antibiotic concentrations during the early stages of cell culture also plays an important role in the inhibition of microbial growth. Further, the medium formulation is a major factor determining the proliferation and lifespan of cultured cells. Since the nutritional requirements of marine invertebrate cells are still unknown, the current cell culture medium used have been mainly designed for vertebrate or insect cell lines[14]. To supply sufficient nutritional materials, pretreated coelomic fluid from sea cucumbers can be used similar to FBS in mammalian cell culture. Meanwhile, to facilitate cell proliferation, several mammalian growth factors can also be applied in culture medium. Since the incubation temperature is a factor impacting the success of marine invertebrate cell culture, incubating cells under the optimal growth temperature of the target organism should be considered. For example, the temperature for *A. japonicus* cell culture can be set at 18 °C, which is suitable for its growth[24].

Tissue pretreatment may impact the successfully cultured and proliferating cell types. Very different *A. japonicus* intestinal cells, round or spindle, can be obtained from seeding filtrated cells or tissue blocks under the exact same culture procedure (comparative data from **Figure 1** and **Figure 3**). Thus, optimizing the tissue pretreating method for specific cell culture type should also be conducted at the beginning; the optimal procedure should be decided according to the biological experimental design.

Here, we applied DXMS treatment for apoptosis induction in *A. japonicus* intestinal cells after three days in culture, and we carried out Hoechst staining for apoptotic signal detection. Successful detection of Hoechst positive cells, after 48 h stimulation by 1 μM DXMS, provided a practical example for a biological experiment based on the cultured cells.

The described protocol is easy-to-handle and can be used for establishing a marine invertebrate cell culture. The cultured cells should provide biological material with consistent genetic background for different further biological experimental applications. Moreover, slightly adjusted protocol procedures can change the successfully proliferating cell types during culture and provide different cell materials for biological experiments. So, the optimization of certain steps in the protocol will be necessary, depending on the targeted animal species and the specific cell types needed.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1 revised

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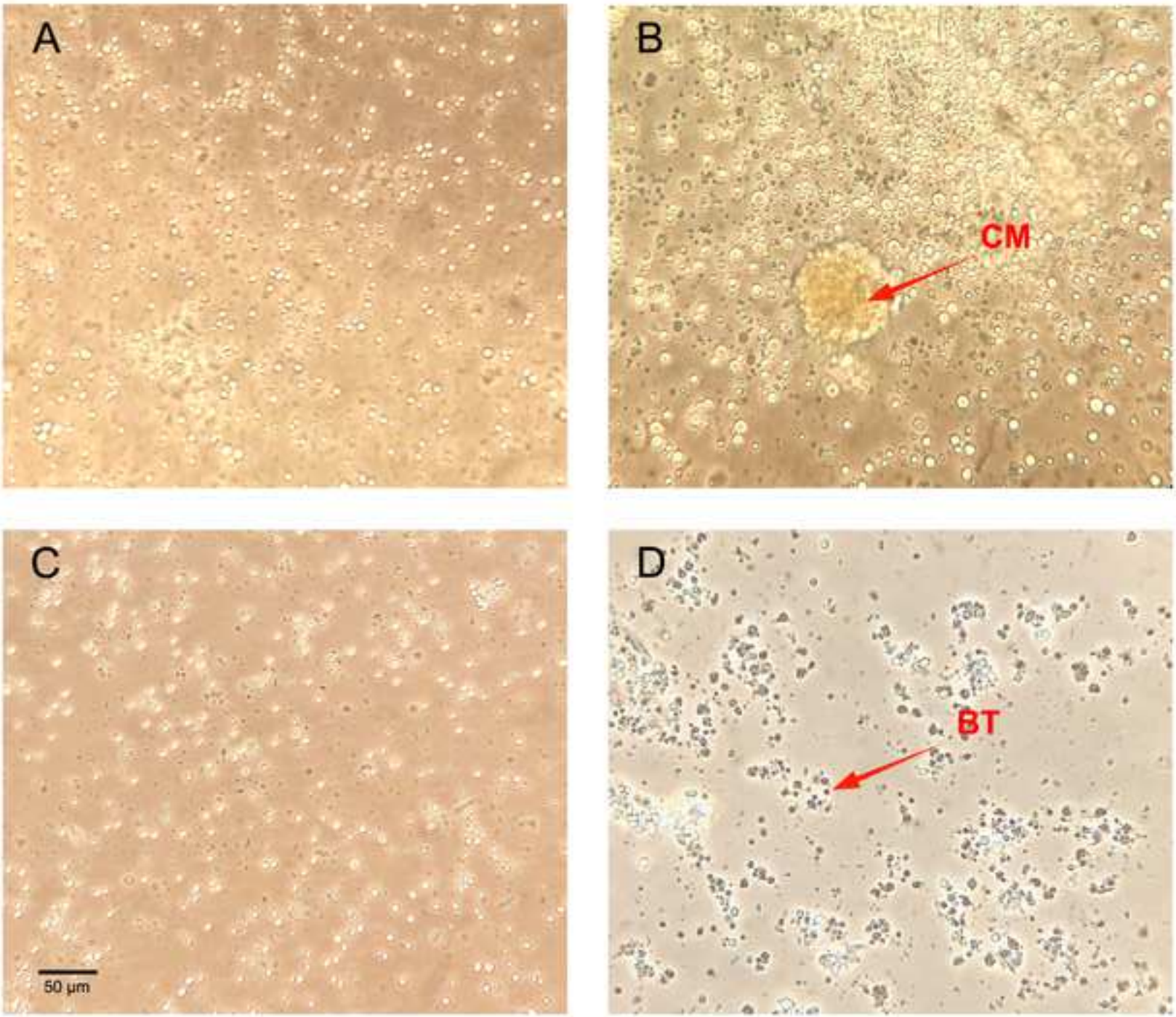

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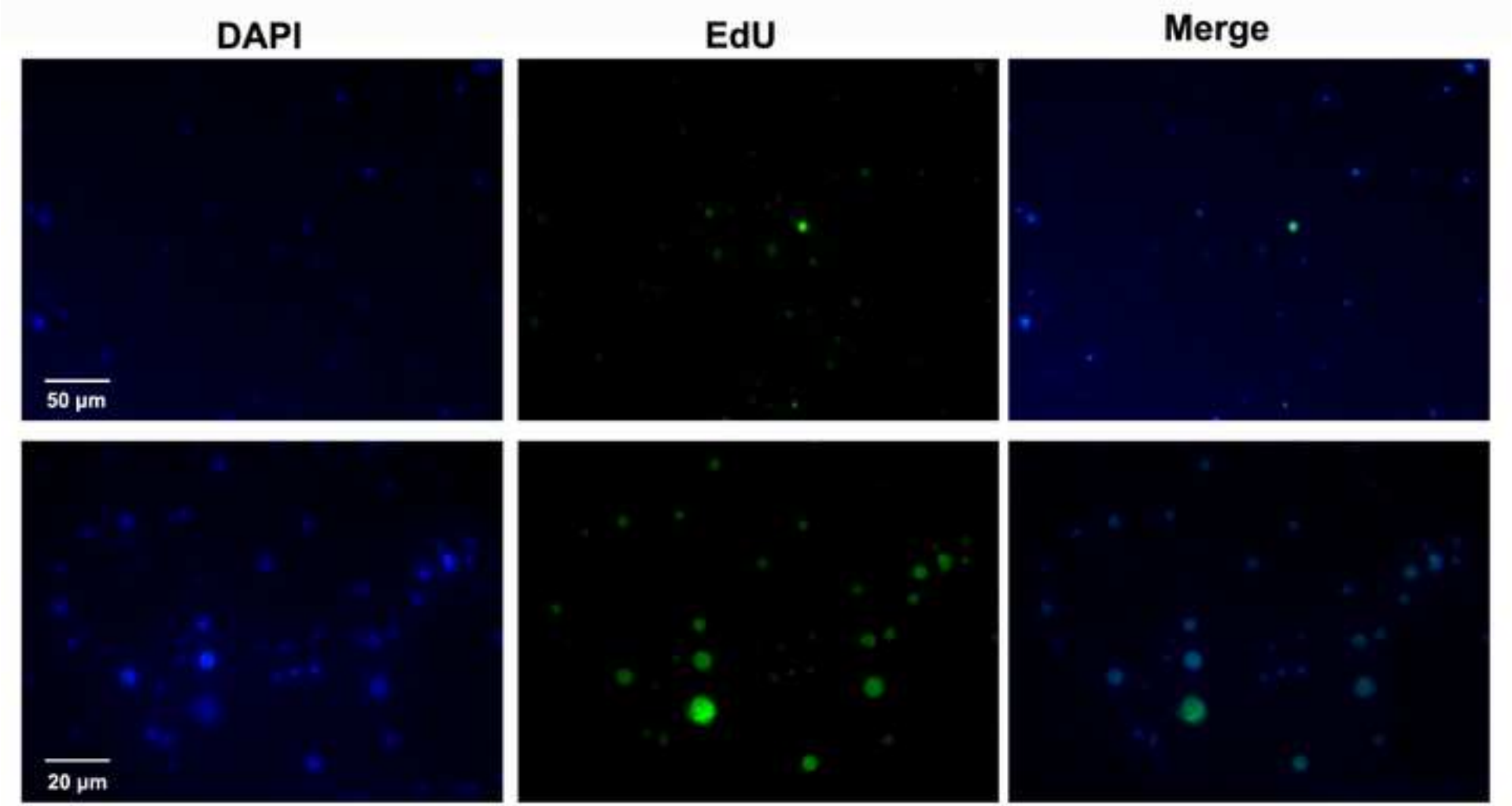

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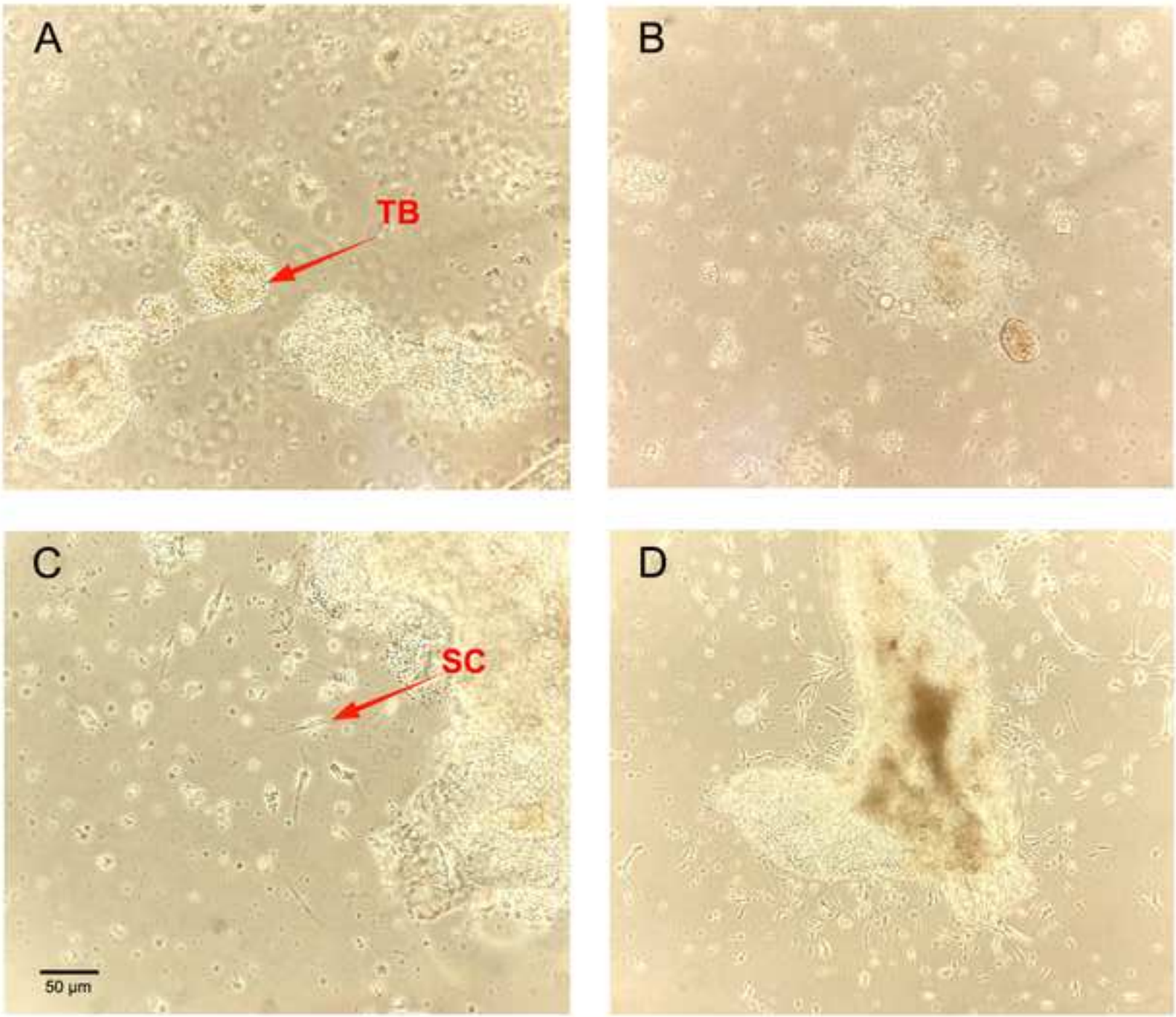

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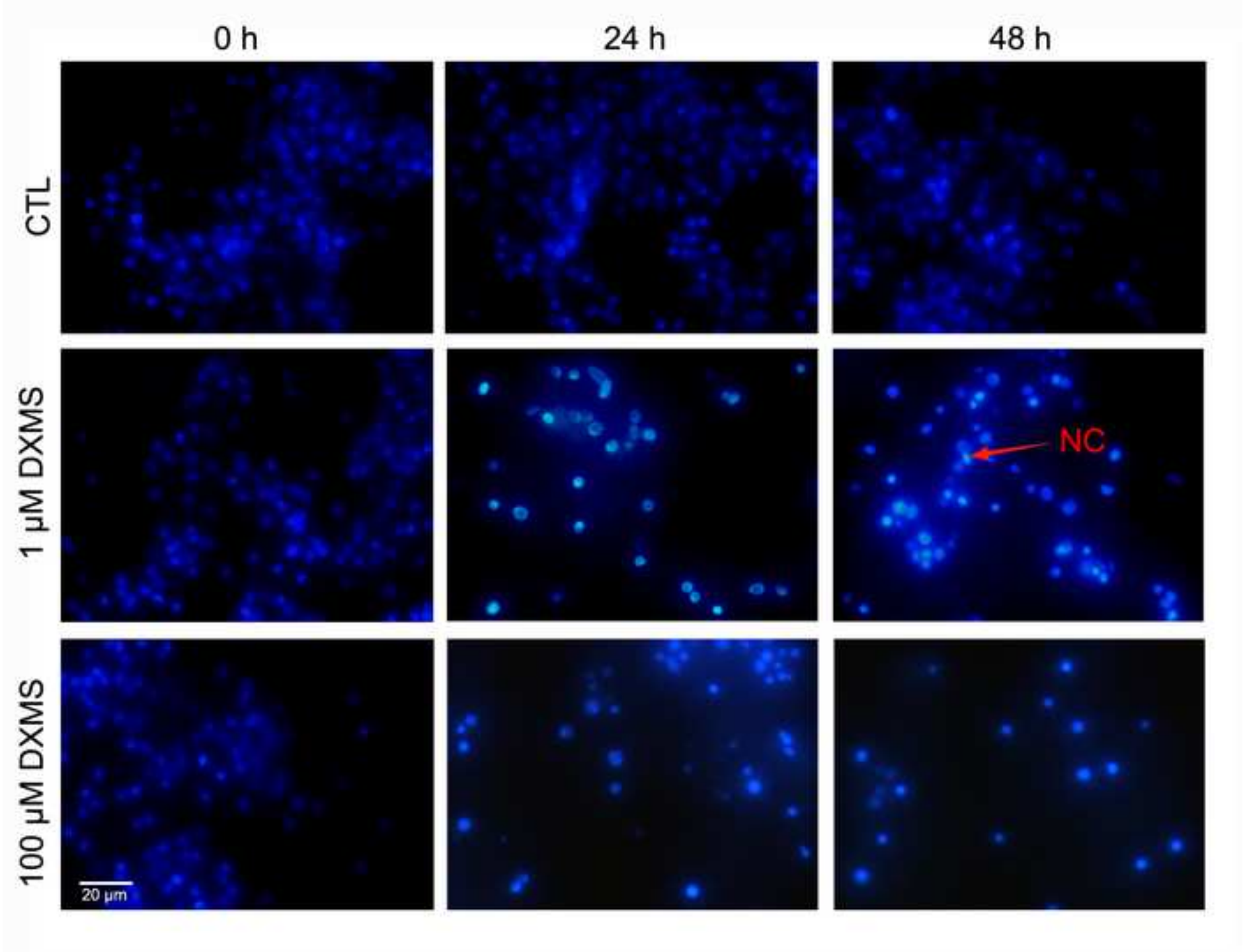

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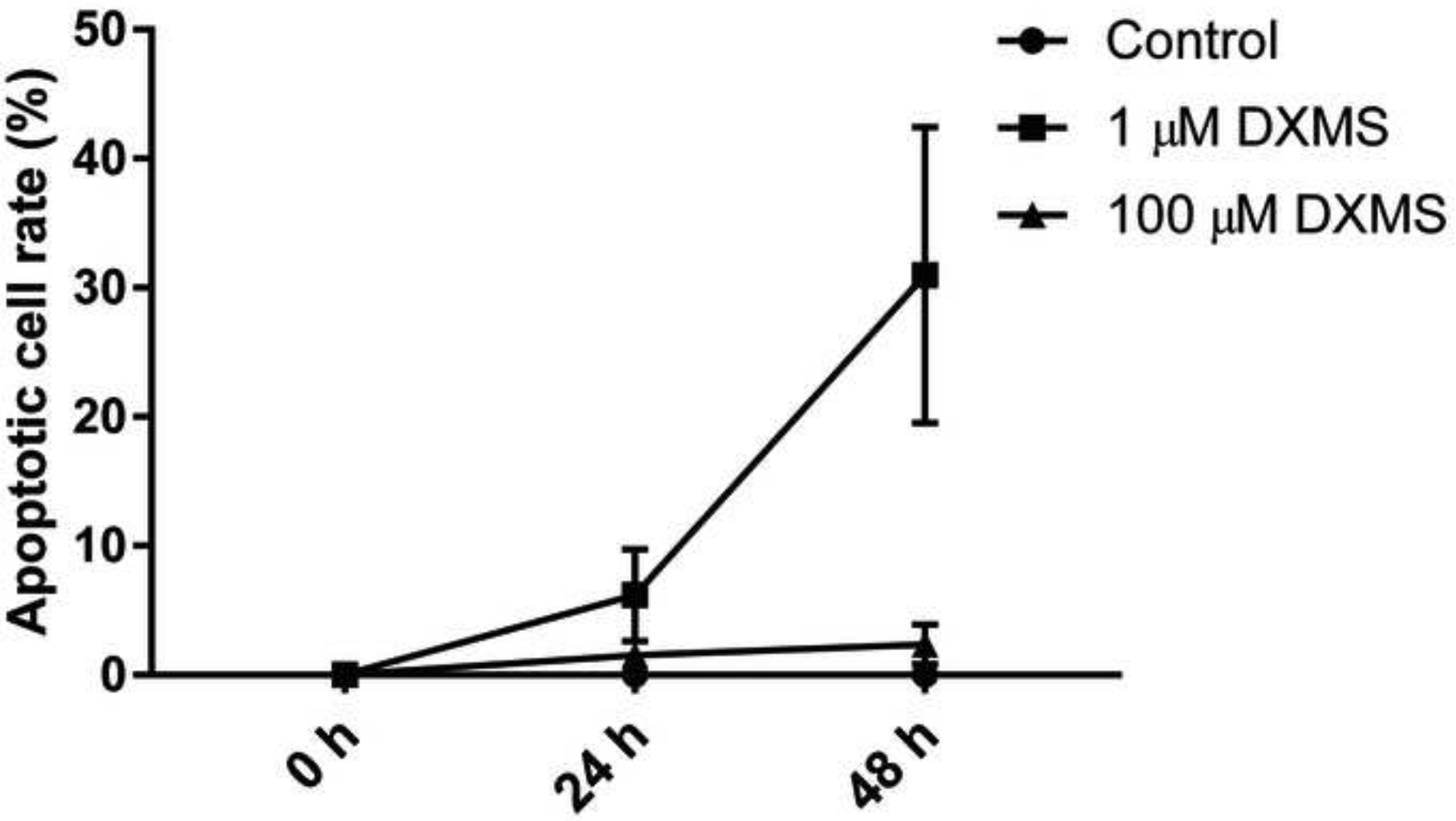

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
0.1 μm filter	Millipore	SLVV033RS	
0.22 μm filter	Millipore	SLGP033RB	
0.25% Trypsin	Genom	GNM25200	
100 μm filter	Falcon	352360	
4 cm dishes	ExCell Bio	CS016-0124	
4% paraformaldehyde solution	Sinopharm Chemical Reage	80096618	in PBS
Benchtop Centrifuges	Beckman	Allegra X-30R	
BeyoClick EdU-488 kit	Beyotime	C0071S	
$CaCl_2$	Sinopharm Chemical Reage	10005817	
Constant temperature incubato	Lucky Riptile	HN-3	
Dexamethasone	Sinopharm Chemical Reage	XW00500221	
Electric thermostatic water bat	senxin17	DK-S28	
Ethanol	Sinopharm Chemical Reage	80176961	75%
Fibroblast Growth Factor(FGF	PEPROTECH	100-18B	
Fluorescent microscope	Leica DMI3000B	DMI3000B	
Garamycin	Sinopharm Chemical Reage	XW14054101	
Glucose	Sinopharm Chemical Reage	63005518	
Hoechst33258 Staining solutio	Beyotime	C1017	
Insulin	Sinopharm Chemical Reage	XW1106168001	
Insulin like Growth Factor(IGF	PEPROTECH	100-11	
KCl	Sinopharm Chemical Reage	10016308	
Leibovitz's L-15	Genom	GNM41300	
L-glutamine (100 mg/mL)	Genom	GNM-21051	
$MgCl_2$	Sinopharm Chemical Reage	XW77863031	
Na_2SO_4	Sinopharm Chemical Reage	10020518	
NaCl	Sinopharm Chemical Reage	10019308	
NaOH	Sinopharm Chemical Reage	10019718	
PBS	Solarbio	P1020	pH7.2-7.4
Penicillin-Streptomycin	Genom	GNM15140	
PH meter	Bante	A120	
Taurine	SIGMA	T0625	
VE	Seebio	185791	

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Author(s): Tianming Wang, Xu Chen, Bing Zhang, Ke Xu, Dexiang Huang, Jingwen Yang

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Dear Editor,

We thank editor for her/his careful reviewing, and we have corrected both the major and minor points to improve our manuscript, and thank editor for her/his suggestions.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response:

The text has been checked and we would like to say that there is no spelling or grammar issue now.

2. Please use h, min, s for time units.

Response:

All the time units have been checked. (Line145, 177, 196, 233, 260)

3. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

Response:

The commercial language was removed. (Line 278, 318)

4. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Response: The trademark and registered symbols were removed both in the manuscript and the table. (Line 318)

Comments-1:

Reviewer #2:
Accept

Response:

Thanks for the reviewer's approval.

Comments-2:

Reviewer #5:
Manuscript Summary:
The manuscript describes a method of apoptosis induction and detection in a primary culture of sea cucumber intestinal cells. The method seems interesting and useful for studying apoptosis process in the similar marine invertebrates.

Major Concerns:
Edited and detailed video recording should be added so that anyone can reproduce the protocol.

Response:

We appreciate the reviewer for her/his careful reviewing, and we were pleased that the reviewer felt that the manuscript is “Interesting and useful”.
According to the publication procedure of JOVE, video will be filmed and produced only after the accepting of the manuscript.

Comments-3:

Reviewer #6:
Manuscript Summary:
The MS titled "Apoptosis induction and detection in a primary culture of sea cucumber intestinal cells" is well written, and overall acceptable. The abstract summarizes exhaustively the protocol and the work, the method for primary culture of intestinal cells from sea cucumber is clearly introduced, and the representative results are quite clear. Overall, this publication is valuable to researchers focusing on marine invertebrate biology. I have some minor comments for the authors to consider.

Minor Concerns:
1. 2.1.2 Wash the tissue samples in phosphate buffered saline (PBS) twice and disinfect them by immersion in an aqueous ethanol solution (75% by volume) for no longer than 2 minutes.
75% ethanol is lethal to the cells. How long the tissue samples were kept in ethanol solution? This information should be accurately provided.

2. 3.4.1 Wash the cultured cells twice using PBS at room temperature.
According to the protocol, the PBS used here may stimulate the cells because of its inconsistent osmoticity to culture medium. Why authors didn't adjust the buffer?

Response:

We thank the reviewer for her/his reviewing and approval. We were pleased that the reviewer felt that “valuable to researchers focusing on marine invertebrate biology”. We have corrected the points to improve our manuscript, and thank the reviewer for her/his suggestions.

The followings are the minor concerns:
1. We thank for the reviewer's careful editing. The time of ethanol treatment here was actually 2 seconds, not 2 minutes. So we changed the time unit. The tissue samples were kept in 75% ethanol for 2 s for sterilization. And this correction was added in 2.12.
2. Actually it is better if the salinity of PBS also adjusted around 30‰. But it is transient for the cells to contact with PBS in the wash procedure. And according to our experiences, there is no significant influence on cells with original PBS compared with the adjusted PBS. Therefore, we use the non-adjusted PBS for protection of trypsin activity and operation convenience. Meanwhile, it is much easier to hadle the normal PBS.

Comments-4:
Reviewer #7:
Manuscript Summary:
The authors have done a remarkable job in clearly depicting the process for extracting and multiplying marine invertebrate cells. Their language is clear, concise, focused and skillfully crafted. In fact, it is perhaps the most clearly written submitted paper I have ever encountered - especially if English is not the authors' first language.
Major Concerns:
No major concerns. This paper will be extremely helpful for years or decades to come, for those investigators following in their path.
Minor Concerns:
None

Response:

We thank for the appreciation from the reviewer. And the spelling and grammar issue was re-checked.

Comments-5:

Reviewer #8:

The paper describes the protocol of a primary culture of sea cucumber intestinal cells. This method could be potentially applied to the culture of invertebrate cells especially in marine creatures. Therefore if this method can be verified effectively and easily handled, it will be a meaningful base for exploration of biological function identification, physiological evaluation and development or immune regulation.

The following issues should be given more consideration and further explanation to improve the quality of the manuscript:

1. We did not see the specific state of the intestine cells in the time course. Could the author give more details of the culture process? "How long did this primary cells last?", for instance.
2. As we all known the intestine is relatively complicated internal milieu, by which evidence that this culture excluded the possible Protozoa from the primary cell they really wanted?
3. The author mentioned that "The described protocol is compatible with a variety of widely available tissue samples from marine organisms including Echinodermata, Mollusca, and Crustacea, ". Are there any figures or data could support this illustration in this study？ If not, please further explain the specific situation.
4. Please further explain the advantages of this method especially compared with the previous work on similar species.

Response:

Thanks for the comments for this reviewer.

1. The primary cells were kept and passaged for 5 months.
2. PCR assay, using actin primers from sea cumumber, was conducted to make sure the cells were from the *A. japonicus* and exclude Protozoa.
3. The method provided in this MS was successfully applied in marine invertebrate species cuttlefish *Sepiella japonica* and crab *Portunus trituberculatus* in our lab. These two species are both non-chordata invertebrates and have adapted to aquatic environment with high salinity, same with echinoderms. However, as the data is under collecting and organizing for a systematic work of one species, we did not show it here.
4. The most important advantage of this protocol is that: the cells from marine invertebrates can be cultured for long time (about 3-5 months) and the cells keep high activity of proliferation in early stages which allows the cell passaging.