SOPHION BIOSCIENCE

Application Report

Cell culturing for automated patch clamp

Ensure a reliable performance of your cell lines by following the Sophion recommendations for cell culturing

Summary

Correct cell handling is important for optimal experimental results. This report gives advice on cell handling in order that you can obtain the best cells possible for use on the QPatch or Qube instruments. Here we describe how to culture your cells and how to reach optimal cell confluence for both cell propagation ("mother flasks") and for experimental use. Detachment enzymes recommended by Sophion are also described together with methods for harvesting and storing cells.

Introduction

High quality cells are critical when performing automated patch clamp experiments, as it is not possible to select which cell that will be used to perform the experiment. It is, therefore, important that the ratio of viable to healthy cells should be as high as possible for an extended period to enable unattended, walkaway operation for a prolonged period.

Sophion application scientists are frequently asked by our users to optimize a specific assay, and often their key parameter for optimization is found to be "pre-patch clamp" and related to the growth, detachment or storage of cells.

Cell lines require stable growth patterns to ensure consistent, reproduceable results. Temperature, CO_2 and culture medium are crucial factors in gaining satisfactory results, but a consistent methodology is equally important and critical to ensure repeatability and reproducibility.

In this application report, we have gathered some of the key points to consider when culturing cells for automated patch clamp. If you still experience problems with your cell culture after following this guide, we will, of course, be happy to assist you further.

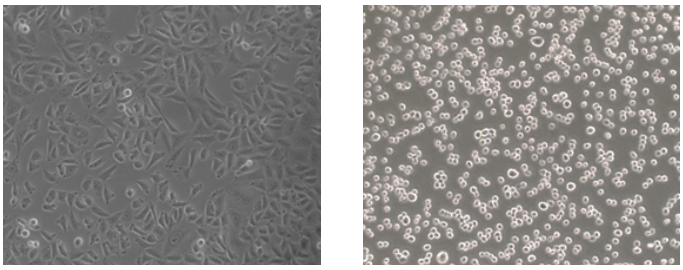
Material and method

Thawing and cultivation

When thawing a new cell line, we recommend starting with a T75 cell culture flask. However, if the cell line is known to show slow growth, a T25 flask could be considered instead (e.g. some HEK cell lines). It is important that the cells are thawed as quickly as possible, e.g. in a water bath at 37°C. Additionally, it is crucial that the cells do not become contaminated, care should be taken that the water doesn't touch the lid of the cryo tube containing the cells. Finally, we recommend spraying the cryo tube containing the cells with 70% ethanol before opening it in the laminar flow hood, where the cells are carefully pipetted into selection-free culture medium pre-warmed to 37°C. After 24h the selection-free medium should be replaced with complete medium (i.e. containing appropriate selection markers). When the cells are approximately 80% confluent you can make the first passage.

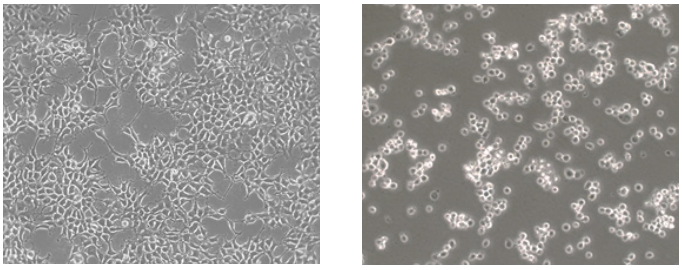
Cell harvest

When cells in the cultivation flask have reached 80% confluence (Fig. 1a, 2a), wash the cells twice in PBS (magnesium and calcium free) and add 2 ml detachment enzyme. Make sure that all cells have been covered by the solution. Remove excess solution until there is only around 0.5 ml solution left. Too much detachment enzyme in your cell suspension may result in poor results. Since almost all detachment enzymes work best at 37°C, place the flask back in the incubator until they are properly detached (Fig. 1b, 2b).



A. B.

Fig. 1: a) CHO hERG DUO ~90 % confluent b) CHO-hERG DUO after trypsinization and ready for harvesting.



A. B.

Fig. 2: a) HEK ASIC, ~80% confluent. b) HEK ASIC after trypsinization and ready for harvesting.

For trypsin, this is usually approximately two minutes (Fig. 1b and 2b). If you don't give the cells enough time they will not form the expected single cell suspension but stay in clusters, whereas too much time will make the cells fragile and success rates may decrease. Tap gently on the flask and add ~5ml culture medium to the flask. Pipette the cell suspension gently up and down 2-3 times. Count the cells either in a hemocytometer (Fig. 3) or with another cell counting device.

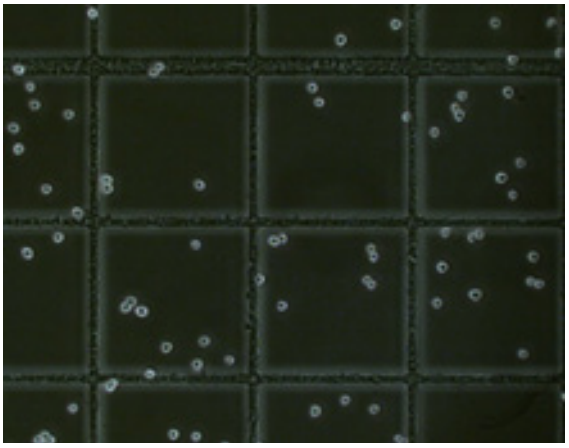


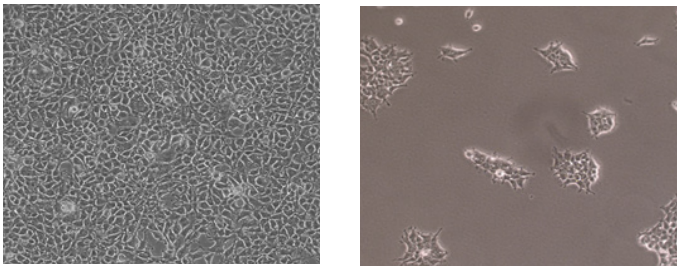
Fig. 3: CHO-hERG DUO in a hemocytometer

Trypsin is a very aggressive detachment enzyme that works well for HEK and CHO cells during passaging and sub culturing. It will keep digesting until it is neutralized. Therefore, don't leave cells for more than 2 min. at 37°C with trypsin. You can either neutralize trypsin with complete culture medium supplemented with serum or Serum Free Medium supplemented with trypsin inhibitor (SFM) (See Solutions for recipe).

Every cell line has its own growth pattern. To ensure that cell culturing is performed in a reproducible manner, we have a specific SOP (Standard Operation Procedure) for all cell lines in the lab. The SOP defines culture medium, seeding and detachment procedure etc.

Keep in mind that the number of cells in the sub-culturing plan (Table 1) may vary. If the cells have an increased or decreased growth curve, you may have to adjust the cell concentration in the cell plan accordingly. We always advise to keep the recommended intervals for passaging the cells to ensure a stable growth pattern.

Depending on which cell line or ion channel you are working with, it may have consequences if your cell line becomes over confluent (Fig. 4a) or too sparse (Fig. 4b). If the cells get over confluent, they may lose expression of the ion channel. If they are too sparse, they might grow in clusters making it difficult to attain a single cell suspension, and this may decrease the seal formation rate on the QChip or QPlate.



A. B.

Fig. 4: Over confluent (a) and too sparse HEK-ASIC cells (b).

Cell culture medium

To avoid human errors, keep a flask of complete culture medium for each cell line (including 100 U/ml penicillin/streptomycin (P/S). We use the full selection medium for both mother flasks and flasks to be used in experiments.

Incubators

The incubators are set to 37°C and 5% CO_2. Temperature stability is the key to achieve a stable growth pattern for the cells. The incubator should only be opened when necessary, ensuring stable temperature and reducing risk of contamination. If possible, we recommend one incubator for mother flasks and a second incubator for flasks used in experiments. This is particularly important if you have a busy cell lab. Failure to maintain a constant temperature will make the cells grow slower and

increase the risk of the cells forming clusters. Remember to keep your incubators clean and tidy.

Passaging/Sub culturing

Be structured: For example, we passage CHO cells on Mondays, Wednesdays and Fridays, whereas HEK cells are only passaged on Mondays and Fridays.

Table 1: Sub-culturing plan for CHO cells.
*48 or 72 hours of sub-culturing are recommended for best results.

Cell type	Confluence target	Time until harvest	Cell concentration
CHO	90%	24 hours	$3.0x10^4$ cells/cm^2
		48 hours*	$1.6x10^4$ cells/cm^2
		72 hours*	$8.0x10^3$ cells/cm^2
		96 hours	$6.0x10^3$ cells/cm^2

Example: The goal is to harvest CHO cells in 48 hours from a T175 flask following the steps below.

1. Harvest the flask.
2. Dilute 100 µl cell suspension with 100 µl trypan blue (T8154 Sigma).
3. Count the mixed cells suspension e.g. with a hemocytometer.

Calculation of cell suspension volume: From Table 1 above, we recommend you seed out $1.6x10^4$ cells/cm^2.

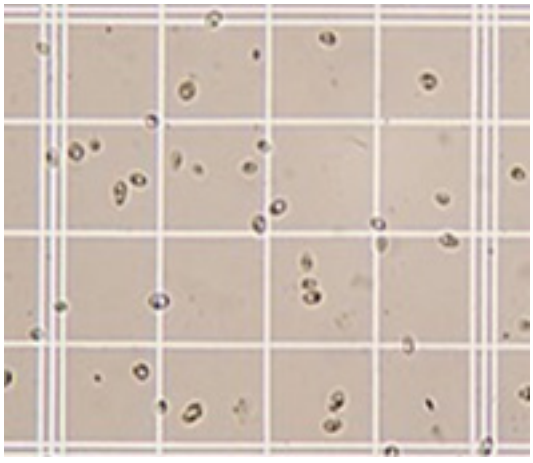


Fig. 5: Counting chamber.

A hemocytometer is separated in 16 squares (Fig.5). The number of cells counted in the 16 squares equals the counted cells x 10^4 / ml. E.g. if you count 100 cells, which are diluted with a factor 2 (trypan blue):

100 cells x 10^4/ml x 2 = $\underline{200 \times 10^4}$ cells/ml

As you aim at seeding 1.6 x 10^4 cells/cm2, the total number of cells to seed out is calculated:

1,6 x 10^4 cells/cm2 x 175cm^2 (T175 flask) = $\underline{280 \times 10^4}$ cells

Consequently, you can calculate the volume that you need to transfer to the culturing flask:

$$\frac{280 \times 10^4 \text{ cells}}{200 \times 10 \text{ cells/ml}} = 1.4 \text{ ml}$$

In summary, you need to add 1.4 ml cell suspension in a T175 flask to 33.6 ml complete culture medium.

Table 2: Sub-culturing plan for HEK cells.
*48, 72 or 96 hours of sub-culturing are recommended for best results.

Cell type	Confluence target	Time until harvest	Cell concentration
HEK	80%	24 hours	$6x10^4$ cells/cm^2
		48 hours*	$3x10^4$ cells/cm^2
		72 hours*	$2x10^4$ cells/cm^2
		96 hours*	$1x10^4$ cells/cm^2

Cell harvesting for experiments

Harvesting of the cells is a critical step for patch clamp experiments. Small changes in protocol, such as over- or under-digestion, can severely affect results and even small details, such as how you tap on a cell culture flask can influence the quality of the subsequent assay.

For harvesting, remove the culture medium, wash twice in PBS and add an appropriate detachment enzyme (e.g. an enzyme from table 3).

Table 3:

Enzyme	Incubation time	Temperature	Cat. No.:
10XTrypsin	2 min.	37° C	T4174- Sigma
Detachin™	3-8 min.	37° C	T100100- Genlantis

Detachment of CHO cells for experiments

For CHO cells, 0.05% trypsin is the primary choice. Trypsin is a very aggressive detachment enzyme that will keep digesting until it gets neutralized. Therefore, watch them regularly while detaching. Normally 2-2.5 minutes at 37°C is enough. Neutralize trypsin with an appropriate amount of SFM (see Solutions) to reach the required cell concentration.

Detachin can also be used for CHO cells, however in our experience there is no gain in cell performance compared to harvesting with trypsin. For busy laboratories with both active HEK and CHO cell lines in the incubators, it is an advantage to use Detachin for both CHO and HEK cells to avoid human errors that can be expensive with respect to both cost and time.

Detachment of HEK cells for experiments

Although trypsin can be used for HEK cells, we would recommend Detachin as the detachment enzyme for HEK cell lines, as we often see an increased viability, less clumping and higher success rates. Some HEK cells are not very well attached to the flask, therefore, it is important to be careful when washing and when adding detachment enzyme to the cells. Pour the PBS on the side of the flask and gently tilt it to make sure the entire cell layer is washed before removing the PBS again. Add 3 ml Detachin and make sure that all cells have been covered. Remove excess solution until there is only around 0.5 ml solution left. Incubation for 3-8 min at 37°C will result in round and well-detached cells. Detachin does not need to be inactivated. Add SFM to the required concentration.

Other detachment options

Success rates, current levels and assay performance in general are normally characteristics related to the specific cell line and target. If none of the standard detachment options described above work for your cell line, try to increase the incubation time. We do not recommend adjusting the concentration or temperature.

There are also other available detachment enzymes that could be tested and optimized e.g.

- Accutase®
- TrypLE

Although we work with most of the commercially available ion channel cell lines, we acknowledge that many companies use proprietary self-developed cell lines. These may need other timings and conditions.

Storage of cells before patch clamp measurement

The cells harvested in SFM can be transferred to a cell hotel and placed on the Qube or the QPatch. Remember to add a stirring bar to the cell hotel. If you want to add cells manually you can place the medium flask on a shaking table to be sure that the cells are in suspension. The durability of the cells depends on which cell line you are working with. Some cell lines, such as TE761, can last all day and others, like HEK-$Ca_V1.2$, only for a few hours.

Freezing cells

Most cell lines lose their expression after a certain number of passages. Some cell lines tolerate only around 10 passages (e.g. $Ca_V1.2$). Others like CHO-hERG-DUO can last up to 40 passages. It is therefore important to note the number of passages on the mother flasks. To keep cell lines, they should be frozen in aliquots and stored in liquid nitrogen. To prepare the cells for freezing, they should be harvested as normal using the FREEZING solution (see Solutions for recipe) as a harvest medium after detaching the cells. Cells are harvested as described previously in aliquots of 4 million cells/ml and frozen down rapidly (1˚C/min) to -80˚C (e.g. Mr. Frosty™). After a week, the cell aliquots should be stored in a liquid nitrogen freezer. It is not recommended to keep cells stored in a –80°C freezer. Cells kept in –80°C freezers for extended periods (months) will show poor growth when thawed and, as a consequence, can lose expression of the recombinant ion channel.

Solutions

Serum free medium (SFM)

25 ml EX-CELL ACF CHO medium (C5467, Sigma-Aldrich, Copenhagen, DK)

25 mM HEPES (H0887, Sigma-Aldrich, Copenhagen, DK)

0.04 mg/ml soy bean trypsin inhibitor (T6522, Sigma-Aldrich, Copenhagen, DK)

100 U/ml penicillin/streptomycin (P0781, Sigma-Aldrich, Copenhagen, DK)

FREEZING solution

90% fetal bovine serum (Tetracycline screened)

10% DMSO (D8418, Sigma-Aldrich, Copenhagen, DK)

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