Technology Note

The Daily Cell Culture

A how-to based on experience

Seeing beyond

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A lot of the medical progress of our time is based on the postulation of cell theory and thus cell biology. The study and research of eukaryotic cells presents a quick and easy opportunity to test metabolic processes, environmental influences or, for example, mechanisms of drug action. Cell biology has become much more complex in recent years and is now able to make statements not only at the single cell level, but also in more structures such as 3D cell culture or organ-on-a-chip applications. The optical magnification of cellular processes and structures using various microscopy methods is indispensable for cell biology. Meanwhile, the race for a resolution down to molecular level has long since begun, with the result that even investigating interactions at the protein level is no longer difficult. However, the cell culture comes at the beginning of every experiment, no matter how complex and along with it comes the microscopy routine. Cell lines have to be produced, maintained and prepared for experiments, and this takes know-how derived from experience. In this technology note, you will get the experience values you need to run a healthy cell culture successfully.

Cell Culture

Cell culture is the basic prerequisite for most experiments in cell biology and also for many industrial applications in e.g. drug screening or the production of various biological products. The advantages of working with cell cultures as opposed to more complex model organisms are obvious. In cell culture, most of the parameters of the experiment are controllable. Physical environmental conditions such as oxygen levels, temperature, humidity etc. as well as the specific treatment can be planned and carried out in a way that is reproducible and completely monitored. In recent decades, a number of highly complex methods have been developed for this purpose, but gold standards have also been continuously refined and optimized.

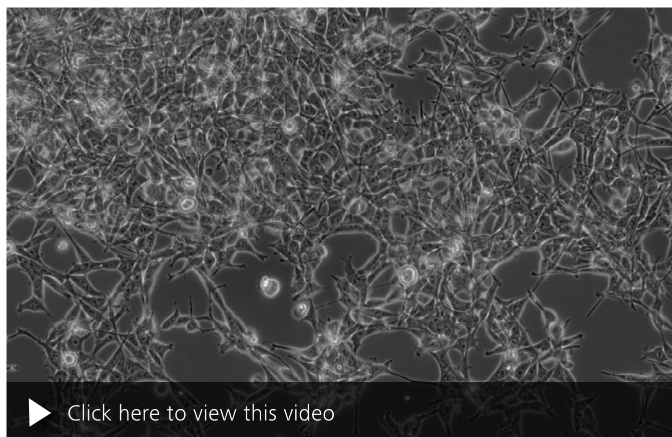

Figure 1 *HEK 293 cells. Long-term time lapse recording of 3 × 3 tiles with 240 s interval. Acquired with Axiocam 506 mono, stabilized by Definite Focus 3 at 10 s interval.*

Cell Culture Methods

Checking the Cell Health

The advantages of cell cultures can only be exploited if you follow certain standardized processes to ensure that the cells are always treated and cultured in the same way. Cells must be cultivated in cell- and experiment-specific culture conditions. These include the type and composition of the culture medium, CO_2 content and temperature, and also the nature of the culture surface or possible co-cultures. To ensure normal physiological growth, cells must be checked every day and their condition must be examined as regards growth rate, cell density in the culture vessel (confluency), unusual morphology and possible contamination. The incubator, the color of the medium and its clarity should be examined with the naked eye. Likewise, possible fungal contamination can often be detected and documented macroscopically. In the second instance, the morphology and the growth need in the vessel should be checked with the help of light microscopic examinations using a magnification between 100× and 400×. It is advisable to image the cells with a contrasting technique such as phase contrast, as the often transparent and very flat cells are hardly visible in simple transmitted light.

To ensure reproducibility for subsequent experiments, it is important to use appropriate methods to mark each culture and manage the data of the investigation. To obtain the most accurate and thus reproducible measurement of the culture characteristics, ZEISS Labscope modules for cell counting and confluency are based on AI to help save time and resources.

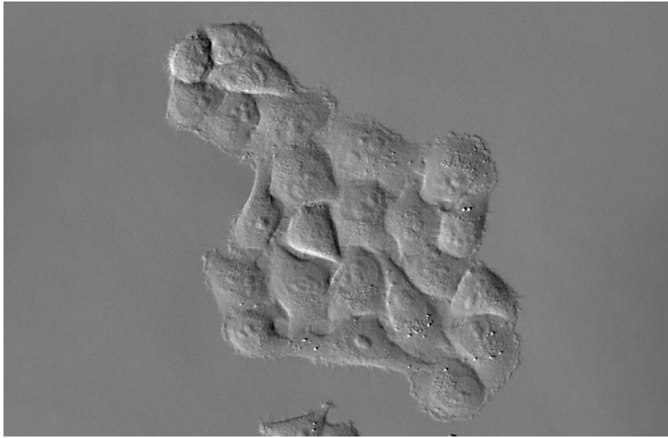

Figure 2 *72h cell growth assay using a waterimmersion objective. HeLa Kyoto cells expressing H2B-mCherry Tubulin eGFP (Neumann et al., Nature 2010 Apr.1.; 464(7289):721-7) imaged every 15 minutes for 72 hours using Autoimmersion; individual channels of the green (eGFP) and red (mCherry) fluorescence and the phase-gradient-contrast as well as an overlay. Sample courtesy of I. Charapitsa, Chemical Biology Core Facility, EMBL, Heidelberg, Germany.*

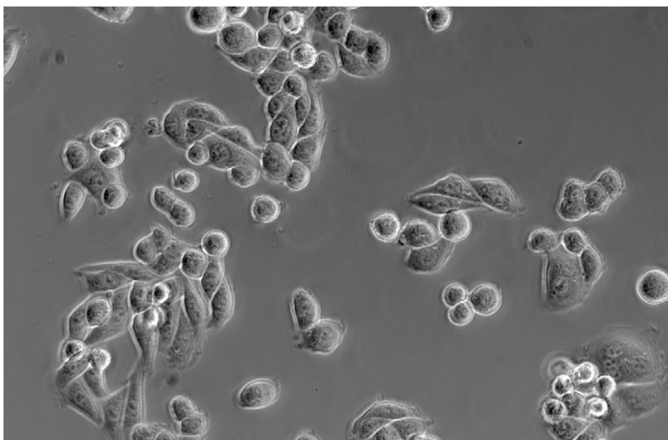

Figure 3 *Slow attachment of freshly seeded HeLa cells to the bottom of the culture dish, phase contrast.*

Once the cells have reached a density of approximately 80 %, it is time to transfer them to a new culture flask since excessive growth will no longer guarantee the cells' supply of nutrients, and the cells’ physiology and metabolism can no longer be regarded as normal in the event of excessive cell contact. However, this is highly cell-specific: some cells actually require precisely this contact to achieve physiological growth.

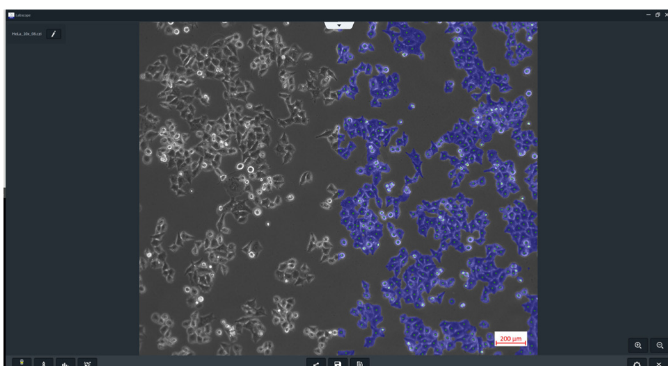

Figure 4 *Cell Counting of eukaryotic cells in phase contrast using AI Cell Counting module in Labscope. Left: uncounted cells in phase contrast. Right: Masked and counted cells after successful cell counting*

Routine Procedure

The daily work in your cell culture lab consists in good part of preparation and care. Every 24 hours, the cultured cells need to be checked and cared for. Yet additionally, every example of lab work has its own preparation and service needs. Before you start handling the cells, make sure all doors and windows are shut. The filters on your laminar flow hood need to be changed regularly. The water in your water bath should also be changed regularly and treated with antimicrobial agents. Keep everything you need at hand, but do not overcrowd your working area.

Protocol for the Daily Cell Check

You will need:

- 70 % technical alcohol
- Sterile gloves
- Inverted light microscope (min. 10× objective + phase contrast)

1. When removing the cells from the incubator, take care not to leave the incubator open any longer than necessary to prevent remaining cultures from cooling down. Try not to move the culture vessels too harshly to avoid the medium touching the filters or lids. Otherwise, contamination bridges can form, enabling contaminations to move from flask to flask.

2. Macroscopic check-up:
   - Has the medium become heavily discolored overnight (pink = too alkaline; yellow = too acidic)?
   - Are clear turbidities visible in the medium?

If so, then there may be contaminations.

3. Microscopic check-up: Image the cells directly in the culture vessel under an inverted light microscope in phase contrast and examine for abnormalities.
   - Are a large proportion of the cells no longer adherent?
   - Are they round and moving with the medium?
   - Do the cells no longer have a uniform morphology?

If so, then the culture parameters may have been unstable or there may be contaminants in the culture.

If possible, discard the cells and replace them with a new culture. If the cells do not behave physiologically, but there is no directly visible reason for this, carry out a check for mycoplasma. DAPI staining and fluorescence microscopy with a magnification of at least 400× are particularly suitable for this purpose.

4. If none of these abnormalities apply to the culture, the cells can be further processed. After a confluence test—for example, by estimating the cell density by eye or, if accurate and reproducible results are desired, by using the ZEISS Labscope AI Cell Counting module—the following options are available:

   - Cell confluency below 80 %: Return cells to the incubator for further growth or, if sufficient cell counts are available, they can be used for experiment preparation.
   - Cell confluency above 80 %: Cells must either be split and passaged into a new culture vessel to be available for further experiments or the cells can be harvested for experiment preparation.

Passaging Adherent Cells

To keep cell lines alive and well in culture, they must be split and passaged into new culture dishes regularly. After a successful cell check, the following protocol is applicable for cell passaging.

Protocol for Cell Passaging

You will need:

- 70 % ethanol
- Fresh cell culture medium
- Phosphate buffered saline (PBS)
- Trypsin-EDTA
- Pipette controller
- Sterile pipettes
- 37 °C incubator (+ cell type specific CO_2 supply)
- New and sterile cell culture vessel

To keep cell disturbance to a minimum, prewarm your liquids in a clean water bath at 37 °C. Before you place the containers in your sterile hood, thoroughly disinfect them by spraying with technical ethanol.

Figure 5 *Proper desinfection of the equipment used under the sterile laminar flow hood with 70 % technical grade alcohol.*

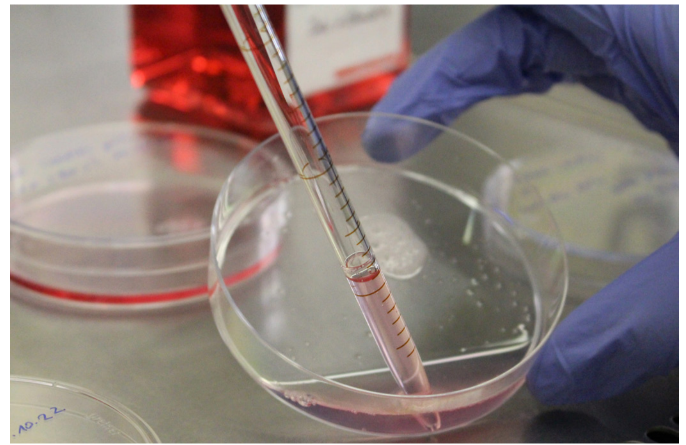

Figure 6 *Removal of dead, unattached cells swimming freely in the growth medium.*

1. Prepare the disinfected working utensils under a sterile laminar flow hood if possible. These include the fresh culture medium, fresh cell culture flasks, sterilized pipettes, a disinfected pipette controller and disinfected racks for Falcon and Epi tubes.

2. Using a pipette, carefully tilt the vessel and remove the complete medium in the vessel to dispose of all dead or floating cells.

3. Rinse carefully with the same volume of PBS as there was medium in the flask and dispose of the PBS in a suitable container. Be careful not to destroy the delicate cell layer at the bottom of the vessel. Do not touch anything outside of your culture flask with the tip of your pipette and take care not to generate a backsplash while discarding the PBS. If you do, use a new pipette for further work.

4. Add Trypsin-EDTA (approximately 20 % of the original volume of the medium, checking the required trypsin concentration for your cell line) to the cells to dissolve the cell layer from the bottom and incubate the suspension for about five minutes at 37 °C (Incubator).

5. Add fresh medium (approximately 80 % of the original medium volume) to the cells. Pipetting the medium-Trypsin-EDTA solution up and down ensures that the entire cell layer is detached. The mechanical effect of the pressure of the pipette jet ensures that all cells are completely detached. The medium stops the effect of the trypsin. Try not to use scrapers since they often disrupt significant amounts of cells and will falsify the cell counting results later. If you doubt whether you have sufficient cells for your experiment, use only 50 % of fresh medium to keep the concentration of your cell suspension high.

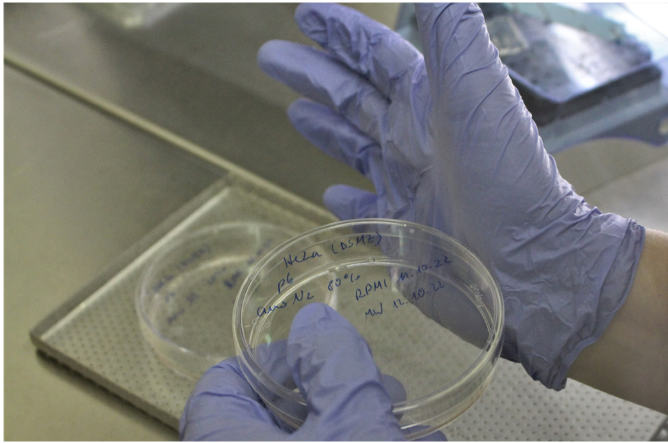

Figure 7 *Gently deattaching remaining cells from the bottom of the dish. Mechanical force supports the proper deattachment process so no cells are wasted.*

6. Check the detachment status via a light microscope. If too many cells are still attached to the culture vessel, try to loosen them by gently moving the flask back and forth or bumping it against your palm. Since different cell lines need different trypsin concentrations to detach successfully from the surface, microscopic monitoring is often recommended for new cell lines.

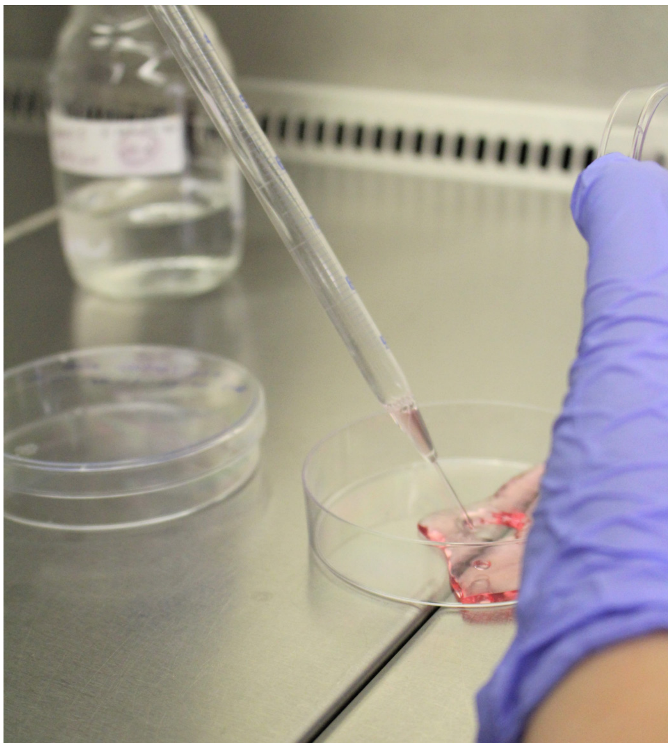

Figure 8 *New culture medium poured with care to avoid contamination from outside the culture dish or the lid.*

7. The cell suspension can now be prepared for further use. To continue the cell culture, fill new cell culture flasks with 1:10 of the cell suspension, topped up with fresh medium and incubated in the incubator at the cell-specific temperature until the next cell check.

8. In case you need higher cell concentration, you need an exact cell number for your experiment or trypsin in your media is unfavorable for your experiments, transfer the cell-trypsin-suspension to a 15 ml or 50 ml centrifuge tube and gently centrifuge the cells with low velocity. Discard the supernatant and resolve the pellet with a specific volume of fresh culture medium. Specific instructions for cell lines can be found in the accompanying documents of the cell lines.

Note: Do not forget to record the cell type, date and your abbreviation on the cell culture flask.

To prepare cells for an upcoming experiment, a defined number of cells usually need to be seeded in cell culture dishes.

Protocol for Seeding Adherent Cells

To seed a defined number of cells, the cell count / ml and total cell count of the culture must first be calculated. Again, an inverted microscope is used for this purpose. For the most accurate measurement, a cell counting chamber is also required.

You will need:

- Inverted light microscope
- Cell counting chamber
- Manual counter
- Micropipettes with tips
- Fresh sterile experiment vessels

1. Using a small-volume micropipette, apply a few µl of the cell suspension to the grid of the counting chamber and cover with the corresponding cover glass. It is advisable to breathe on the cover glass briefly beforehand so that it sticks to the counting chamber later.

2. You can now count the number of cells of the 16 squares under the microscope with phase contrast according to the diagram below (Diagram 1).

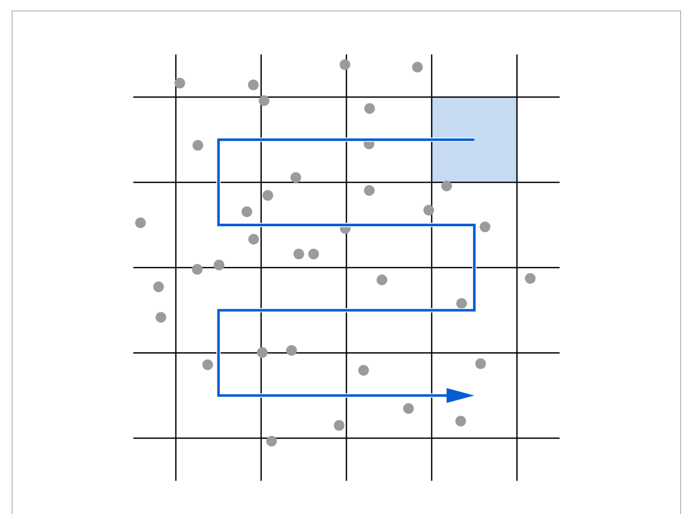

Diagram 1 *Counting pattern for cell counting chambers.*

Then use the following formula to obtain the number of cells per milliliter:

$$\text{Total cells/mL} = \frac{\text{Total cells counted} \times \text{dilution factor} \times 10{,}000 \text{ cells/mL}}{\text{\# of counted squares}}$$

The dilution factor is 1, as the cell suspension has not yet been filled with medium. If diluted cell suspension has already been used for cell counting — for example, as mentioned above for passaging in a ratio of 1:10 — the factor 10 should be used here.

3. Once the correct cell count has been defined, you can mix the required volume of cell suspension with cell culture medium and pipette it into the experiment vessel. For the most even distribution of cells, try sliding the culture vessel back and forth in a random motion flat on the bench. There should be as few waves as possible. The cells should again be given at least 24 hours in the incubator to grow and assume their physiological behavior before the experiment. For experiments with defined volumes, use the formula: Volume for dilution = "what concentration do I want" divided by "what concentration do I have" times "desired final volume".

Recommended microscope equipment:

- Inverted light microscope with brightfield and phase contrast
- Objectives: 10×
- Various stage inserts for different cell vessels

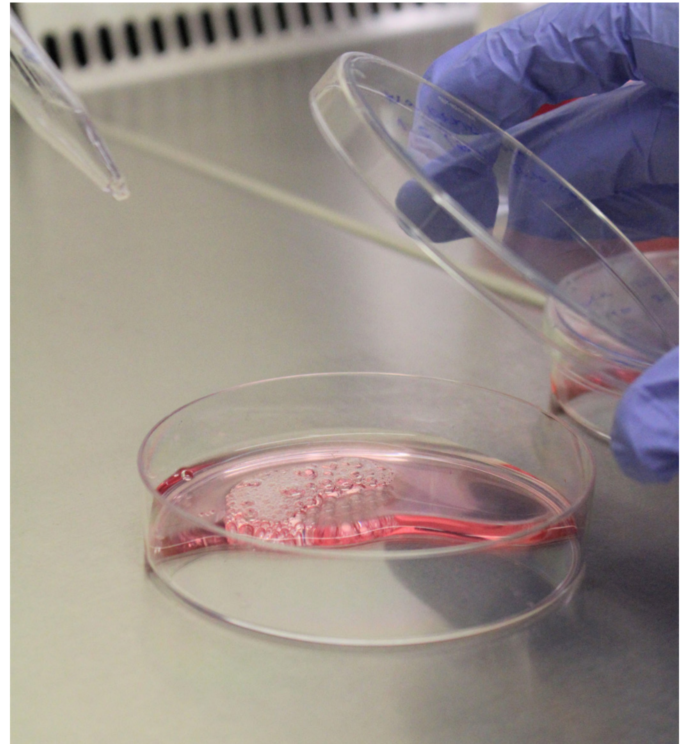

Figure 9 *Keep the lid slightly over the culture dish and quickly close the lid after seeding to avoid contaminats.*

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