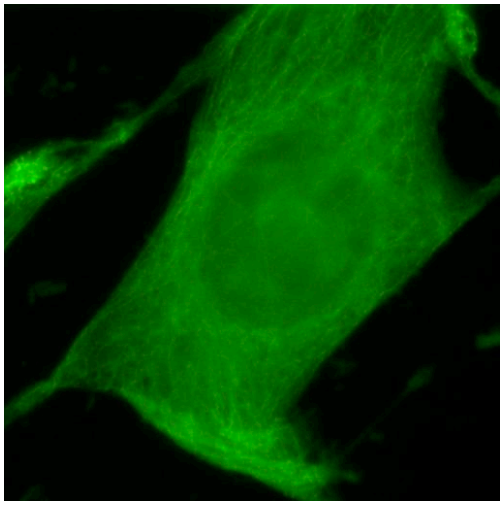
Laborationskompendium
Livsvetenskaplig grundkurs (BI0779)
Tema 2: Cellen och Genen



Bilden på omslagssidan visar mikrotubuli i mammal celler. Mikrotubuli är visualiserade med hjälp av anti-tubulin antikroppar (jämför laboration två, Mikroskopi, i detta häfte).

Laborationsrapporer skall skrivas för alla tre laborationerna. Dessutom kan era handledare komma med ytterligare ändringar och tillägg. Om rapporten lämnas in i tid och blir godkänd på första försöket fås bonuspoäng på sluttentamen.

Laboration	**Lämna in**	**Handledare**
Cellodling	23 Februari 2009	Tom Martin,
Mikroskopi	2 Mars 2009	Anna Johansson,
Celldifferentiering-celldöd	16 Mars 2009	Daniel Uddenberg

Hör av er till mig eller era handledare om ni har frågor eller funderingar.

Lycka till!
/Peter (Peter.Bozhkov@vbsg.slu.se)

Protokollen i detta kompendium uppdaterades January 2009 av Jenny Carlsson, Anna Johansson, Tom Martin, Daniel Uddenberg, Peter Bozhkov

CELL CULTURE

Time: Two half days			
Risk class:			
☐No risk	☑Low risk	☐Medium risk	☐High risk
Risk type: Chemicals			See also box at the end of lab

Introduction

Cells are normally grown in a medium containing an energy source, minerals, organic molecules for biosynthesis, vitamins and a buffer with a pH sensitive dye. Most eukaryotic cells also require specific stimulatory growth factors. These are usually supplied by the addition of serum (which may contain a lot of different growth factors) or more rarely by the addition of purified such factors. To some extent though, the cells themselves may actually also secrete them into the medium. Since the growth media are excellent sources of nutrition for almost any cell type, contamination of cell cultures with microorganisms must be strictly avoided, since they otherwise will rapidly overgrow the cells due to their more rapid growth rate. It is thus essential to maintain sterile conditions and all handling should be done in a laminar flow hood or other hoods with sterile filtered air. All solutions and material coming into contact with the cells must also be kept sterile. The cells will divide if the conditions are favourable but many cells may take up to 20 h or even more between divisions. Cells are usually grown in bottles, dishes or multi-well plates made of disposable plastic. The cells can be free (suspension) or stick to the plastic (adherent cells). The plastic can be treated with different compounds (collagen, fibronectin, polylysine etc.) to get the cells to attach more firmly if this is required. When the cells have divided several times the density becomes too high and the nutrients become limiting. When this occurs the cells may be "split" - i.e. diluted into fresh medium. The growth/proliferation then continues. For adherent cells the cells are released by the addition of EDTA or trypsin, which disrupts the extracellular matrix “glue”. A suspension culture is simply diluted into fresh medium.

In this practical you will get acquainted with propagating cells in cell cultures. You will learn basic techniques for handling of cells. You will also learn how to take appropriate measures to ensure that the cultures do not get contaminated by microbes (i.e. sterile technique). Specifically for this lab, you will study the stimulatory effect of serum addition to cells. Serum contains a multitude of cell specific growth factors which in many cases have been shown to stimulate cell proliferation already at very low concentrations. The principal means of showing the serum dependence in this particular lab is to study the amounts of protein as a function of serum concentration. However, you will also be able to follow the cell cultures by studying cell morphology (e.g. study what happens to cells during trypsinisation and as they attach to the growth substratum) and by seeing how cell numbers increase during proliferation.

Overview of experimental procedure

Day1

Trypsinise pre-grown cells; study cells under the microscope to try to see what happens during trypsinisation. Establish test cultures with varying amounts of serum added and determine the initial number of cells by counting in a hemocytometer (appendix 1).

Solutions and materials:

- Cultures of adherent HeLa cells
- 70% Ethanol
- DMEM (Dulbecco´s Minimal Essential Medium)
- Fetal calf serum
- Trypsin/EDTA
- Trypan blue solution
- PBS
- 0.5 M NaOH
- Plastic bottles, plates and multi-well plates.
- Sterile pipettes.

Day 2

Determine the overall morphology of well spread adherent cells in the different serum concentrations. Make sure they are doing alright and that the cultures have not been infected. Determine the amount of protein in the cells, as a quantitative measure of how growth and proliferation is stimulated by addition of serum. Estimate cell proliferation with respect to the number of cells for each serum concentration.

Solutions and material:

- Pure water (MilliQ)
- 0.5 M NaOH
- PBS
- Trypsin/EDTA
- Bio-Rad Dye Reagent (store at 4 ºC)
- 0.1% BSA (1 mg protein/ml), calibrate the solution by reading the absorbance at 280 nm where 1% BSA (10 mg protein/ml) corresponds to A_{280} 6.6
- ELISA spectrophotometer

Experimental procedure

Sterile routines for work in the cell lab

- Wash your hands before starting your work.
- Sanitize the cabinet using 70% ethanol before commencing work.
- Sanitize gloves/hands by washing them in 70% ethanol and allowing to air dry for 30 seconds.
- Equipment in the cabinet, or that brought into the cabinet, during cell culture procedures (media bottles, pipette tip boxes, pipette aids) should be wiped with tissue soaked with 70% ethanol prior to use.
- Put all materials and equipment into the cabinet prior to starting work.
- Avoid keeping the incubator door open for longer than necessary and close it very carefully.
- Whilst working do not contaminate gloves by touching anything outside the cabinet (especially face and hair). If gloves become contaminated re-sanitize with 70% ethanol as above before proceeding.
- Movement within and immediately outside the cabinet must not be rapid. Slow movement will allow the air within the cabinet to circulate properly.
- Speech, sneezing and coughing must be directed away from the cabinet so as not to disrupt the airflow.
- After completing work, spray the work surfaces inside the cabinet with 70% ethanol and wipe dry with tissue.
- Cell culture should be discarded in jodopax prior to discarding down the sink.

Day 1

Seed cells in growth medium containing different concentrations of serum

In this lab you will use HeLa cells (a human cancer line), a commonly used cell line. Each group will get one 10 cm dish of pre-cultured HeLa cells. Proceed according the following protocol:

1. Clean the hood, wipe with 70% ethanol.
2. View cultures using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants.
3. Remove the medium by pipetting close to the edge of the plate.
4. Wash (add carefully to the edge of the dish, swirl around, then remove) the cell monolayer with 5 ml prewarmed PBS.

5. Pipette 1 ml prewarmed trypsin/EDTA onto the washed cell monolayer. Rotate the dish to cover the monolayer with trypsin.
6. Incubate the dish for 2-10 minutes. Check every now and then under the microscope to see what happens when cells start to detach. Do not incubate more than 10 minutes!
7. Examine the cells using an inverted microscope to ensure that all the cells are detached and floating. The side of the dish may be gently tapped to release any remaining attached cells.
8. Add 4 ml of medium *without* any serum (0%) to the cells. Pipette up and down, carefully so that no air bubbles form. Rinse the bottom of the plate making sure that all cells have detached. It is really important that there are no cell clumps and that you have a homogenous single cell suspension.
9. Take one 6-well plate and one 96-well plate and add 2000 µl and 200 µl respectively of medium with different serum concentrations to each well (0, 0.5, 1, 5, 10, 20 % serum). Mark each well with the serum concentration. Write your name on both plates

0%	0,5%	1%
5%	10%	20%

0%	0,5%	1%	5%	10%	20%						
0%	0,5%	1%	5%	10%	20%						
0%	0,5%	1%	5%	10%	20%						

10. Add 100 µl of the cell suspension to the each well on the 6-well plate and 10 µl cell suspension to the assigned wells on the 96-well plate. It is important that the cell suspension is thoroughly mixed before distributed on the corresponding plate.
11. In an eppendorf tube add 20 µl trypan blue. Prepare the hemocytometer (step 1, below). Add 20 µl of your cell suspension (from the large 10-cm dish?) to the trypan blue and pipette up and down to mix. Trypan blue is a dye that will stain dead cells blue. Living cells will actively pump out the dye and can thus be seen as bright blobs in the microscope.
12. Count the cells using a hemocytometer (see instructions below and Appendix 1) to determine starting cell density.
13. Incubate both plates in the incubator (37 ºC and 5% CO_2)
14. Clean the hood, wipe with 70% ethanol. Pour some jodopax into the remaining cells, empty in the sink and throw the plate into a “riskavfall” box.

Key Points

1. Trypsin is inactivated in the presence of serum. Therefore, it is essential to remove all traces of serum from the culture medium by washing the monolayer of cells with PBS
2. Cells should only be exposed to trypsin/EDTA long enough to detach cells. Prolonged exposure could damage surface receptors.

How to use a hemocytometer

1. Moisten the cover-slip edges with water. Slide the cover-slip over the chamber back and forth using slight pressure. Make sure the pattern of the chamber is covered by the cover-slip.
2. Fill both sides of the chamber with approximately 10 µl of trypan blue/cell suspension mix and view under a light microscope.
3. Count cells in at least 3 squares (a diagonal) surrounded by triple lines (see appendix 1). Decide how to treat cells on the border and make sure you don't count them twice. Only count the viable cells (seen as bright cells), non-viable cells are stained blue. If you do not have the correct focus all cells will appear dark, change the focus slightly until the majority of cells appear bright. Ideally >100 cells should be counted in order to increase the accuracy of the cell count
4. Divide the total number of cells counted with the number of squares counted. This gives the average cell number per square. Multiply this with 2 (correction for the dilution with trypan blue). Multiply that number with 10000. This is the number of viable cells per ml in your original cell suspension.
5. Clean the hemocytometer and cover-slip first with deionized water, then with 70% ethanol. Wipe them dry carefully.

Key Points

1. Trypan Blue is toxic and is a potential carcinogen. Do not breathe the vapor.
2. There are several sources of inaccuracy for the hemocytometer:
    - The presence of air bubbles and debris in the chamber
    - Overfilling the chamber such that sample runs into the channels or the other chamber
    - Incomplete filling of the chamber
    - Cells not evenly distributed throughout the chamber
    - Too few cells to count. This can be overcome by centrifuging the cells, resuspending in a smaller volume and recounting.
    - Too many cells to count. This can be overcome by using a higher dilution factor in trypan blue e.g. 1:10

Day 2

Measure protein concentration and count cells

Since the cells will be killed you don't have to work under sterile conditions.

Cell counting

Take out your 6-well plate from the incubator. What do you see? You are going to count the cells in the hemocytometer again. Use one concentration on each side of the hemocytometer (count two samples at the same time then clean the hemocytometer before counting the next two samples).

1. Remove the medium from the wells you are going to count.
2. Wash with 1 ml PBS (add carefully to the edge of the dish, swirl around, then remove).
3. Add 200 µl trypsin and incubate for 2-10 min.
4. When the cells have detached from the plate, add 1 ml medium to each well. Pipette up and down and rinse the bottom of the well
5. Count the cells as on day one. Calculate the total number of cells in each well (not the concentration?).

Protein determination using the Bio-Rad Protein assay method

The Bio-Rad Protein assay, based on the method of Bradford, is a simple and accurate procedure for determining concentration of solubilized protein. It contains an acidic dye solution that binds to proteins, primarily basic and aromatic amino acid residues and especially arginine. The absorbance maximum for the dye solution shifts from 465 nm to 595 nm when binding to protein occurs. A spectrophotometer (ELISA) is used to read the absorbance at 595 nm. Comparison to a standard curve provides a relative measurement of protein concentration. The protein concentration can further be used as an approximation of cell proliferation.

The protein concentration of a test sample is determined by comparison to that of a series of protein standards known to reproducibly exhibit a linear absorbance profile in this assay. Although different protein standards can be used, we have chosen the most widely used protein as our standard - Bovine Serum Albumin (BSA).

1. Prepare a dilution of the dye reagent by adding 1 part Dye Reagent Concentrate with 4 parts Milli-Q water. Filter through a Whatman #1 filter (or equivalent) to remove particles.
2. Prepare the protein standard by adding 1 part BSA and 2.5 parts Milli-Q water.
3. Remove the media from the cells and wash each well of the 96-well plate 3 times with 200 µl PBS. It is important that the PBS solution is thoroughly removed before the next step.
4. Add 35 µl 0.5 M NaOH to the cells and let it react 5-10 minutes with the sample.
5. Add 200 µl Dye Reagent to each sample.

6. To set up the protein standard curve you take the amount of BSA and then add H_2O up to 35 µl. The amount of BSA added to the wells; 0.5, 1, 5, 10, 20, 25, 35 µl (1 µl= 1 µg protein).
7. Add 200 µl Dye Reagent to each well containing BSA
8. Read the absorbance at 595 nm within 1 hour. All bubbles in the wells have to be removed.

0%	0,5%	1%	5%	10%	20%						
0%	0,5%	1%	5%	10%	20%						
0%	0,5%	1%	5%	10%	20%						
H_2O	0.5µg	1 µg	5 µg	10 µg	20 µg	25 µg	35 µg				
H_2O	0.5µg	1 µg	5 µg	10 µg	20 µg	25 µg	35 µg				
H_2O	0.5µg	1 µg	5 µg	10 µg	20 µg	25 µg	35 µg				

Plot the absorbance against the amount of protein to establish a BSA-standard curve. The protein content of the different test samples can then be calculated from this standard curve by reading the protein amount that corresponds to the recorded absorbance value. The values should be in the linear range of the standard curve to be reliable.

Questions

1. Why is serum used in cell culture medium? One aspect is not discussed in the introduction.

   Hint: How did the cells look when grown in 0% and 0,2% serum compared to the other wells? How did the cells look during the trypsinization?

2. Why is fetal calf serum used, and not serum from calves or adult animals?

   Hint: search google for words such as: fetal, adult, serum, “tissue culture”, “cell culture”

3. What are the advantages and disadvantages with mammalian cells as a model system?

   For example it is easy to use compared to doing tests in animals, but it is more complicated than culturing bacteria.

References

Cellodling. En praktisk handledning i odling av mammalieceller. Kielberg, Brünner, Briand. Labassco 1994.

Animal cell culture - a practical approach. Freshney. IRL Press, 1987.

Risks and protection

Chemicals:
•

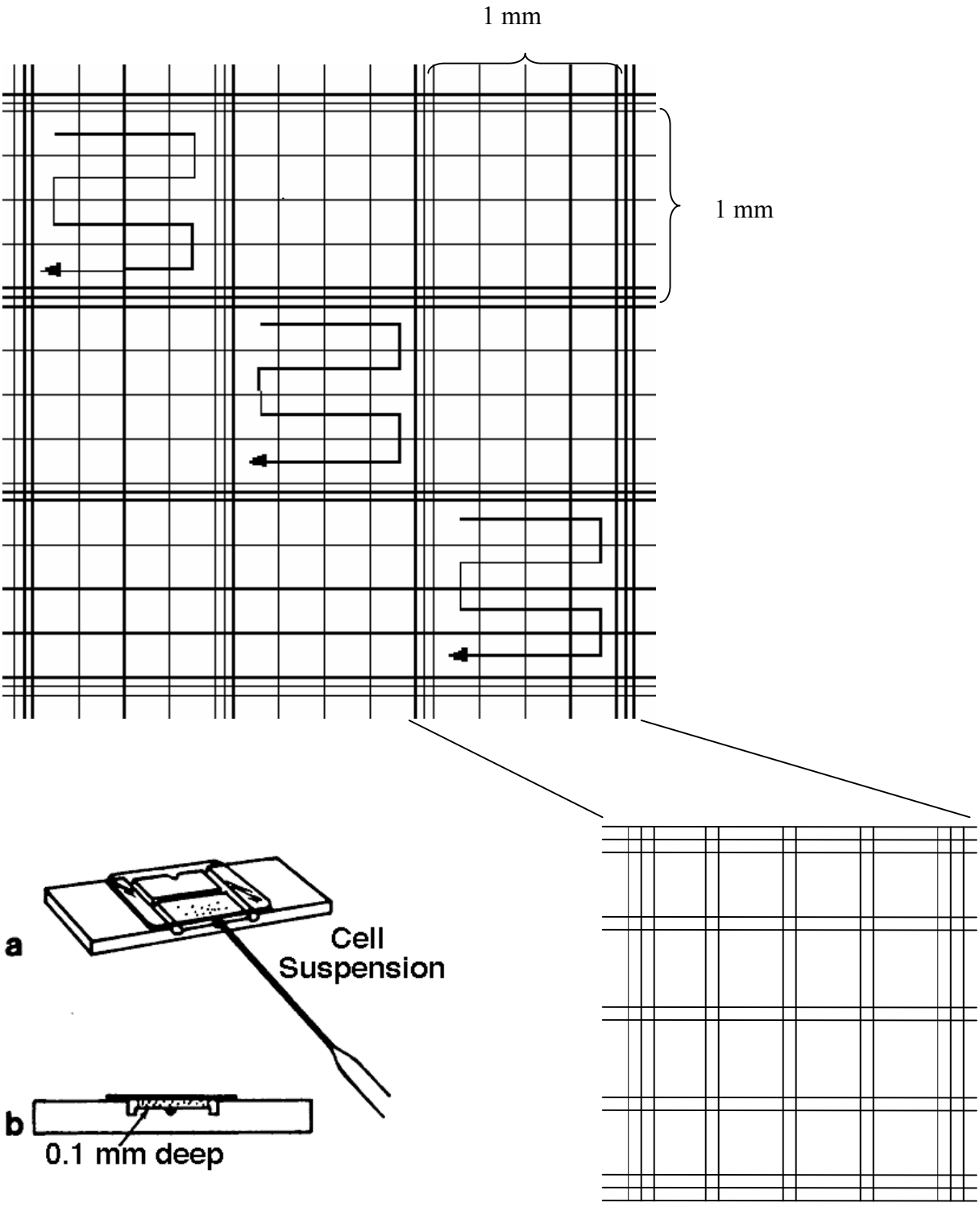
Organisms:
• Not applicable

Radioactivity:
• Not applicable

Other:
•

Appendix 1

***Hemocytometer (Bürker chamber)**:*



Appendix 2

Dulbecco's Modified Eagle Medium (Gibco Catalogue Number: 11965)

DMEM COMPONENTS	Conc. (mg/L)	Molarity (mM)
INORGANIC SALTS:		
Calcium Chloride ($CaCl_2$)	200	1.80
Ferric Nitrate ($Fe(NO_3)_3$-$9H_2O$)	0.10	0.000248
Potassium Chloride (KCl)	400	5.30
Magnesium Sulfate ($MgSO_4$)	97.67	0.813
Sodium Chloride (NaCl)	6400	110.34
Sodium Bicarbonate ($NaHCO_3$)	3700	44.10
Sodium Phosphate (NaH_2PO_4-H_2O)	125	0.906
OTHER COMPONENTS:		
D-Glucose	4500	25.00
Phenol red	15	0.0346
AMINO ACIDS:		
L-Arginine-HCl	84	0.398
L-Cystine 2HCl	63	0.200
L-Glutamine	584	4.00
Glycine	30	0.399
L-Histidine HCl-H2O	42	0.20
L-Isoleucine	105	0.802
L-Leucine	105	0.802
L-Lysine-HCl	146	0.798
L-Methionine	30	0.201
L-Phenylalanine	66	0.400
L-Serine	42	0.400
L-Threonine	95	0.078
L-Tryptophan	16	0.078
L-Tyrosine 2Na $2H_20$	104	0.398
L-Valine	94	0.803
VITAMINS:		
D-Ca pantothenate	4	0.0083
Choline Chloride	4	0.0285
Folic Acid	4	0.00906
i-Inositol	7.2	0.04
Niacinamide	4	0.0328
Pyridoxine HCl	4	0.0196
Riboflavin	0.4	0.00106
Thiamine HCl	4	0.0118

Ref:Dulbecco, R. and Freeman, G. (1959) *Virology* **8**:396.

IMMUNOCYTOCHEMISTRY

- a theoretical lab practical

Note! This lab practical is performed as a dry-lab using the material provided in the folder *students_immunocytochemistry*. Still you will go through it as if you were doing the wet-lab yourselves. Instead of a traditional lab report you will hand in a questioner. Even if you are allowed to cooperate you should hand in one questioner per person. As you will see, the protocol has the same appearance as a normal wet-lab protocol. It should be possible to find many of or most of the answers in this protocol, your course literature or the enclosed articles. Sometimes you can relay on previous knowledge or you should speculate. You should answer the questions in English (simply fill in your answers in the questioner and send them by e-mail to your teacher). Your teacher(s) may suggest changes in the questioner *etc.*, so up-date your task accordingly. If it is difficult to find signals in a certain micrograph (photography from the microscope) in a print-out, study the micrograph on the screen (*e.g.* zoom in).

You need four documents (should be found in the folder *students_immunocytochemistry*);

protocol.pdf	the protocol which has been used in this lab practical
questioner.pdf	questions that you should answer and hand in
Hoglund_1991_immunolokalisering.pdf	research article, from which this lab practical is based
Hoglund_1992_immunolokalisering.pdf	research article, from which this lab practical is based

CELL VIABILITY AND DEATH

In situ detection of programmed cell death in plant embryos

Time: 3 hours or half a day
Risk class:
☐No risk ☑Low risk ☐Medium risk ☐High risk
Risk type: Chemicals, ultraviolet light See also box at the end of lab

Introduction

Aims

The aims of this laboratory practical are to detect programmed cell death (PCD) using nuclear staining and to understand why PCD is vital during the development of an organism. Read about PCD in *e.g.* your course literature, at http://vaxt.vbsg.slu.se/~peterb/ and in scientific papers and other course books.

About model system

In this practical you will learn how to detect programmed cell death (PCD) ***in situ*** (*i.e.* exactly in place where it occurs) in a multicellular model system – plant embryos. Norway spruce (gran, *swed.*) embryos are an ideal model for PCD, due to the easy distinction between the living and the dying parts of the embryos and their large size. Furthermore, embryogenesis in Norway spruce can be induced to start from somatic cells in laboratory conditions thus enabling to obtain large quantities of genetically and phenotypically similar embryos for different assays. The embryos obtained in such a way are called **somatic embryos** emphasizing their somatic rather than zygotic origin. Accordingly, the process of embryo development from somatic cells is called **somatic embryogenesis** (Figure 1). In Norway spruce, somatic embryogenesis resembles zygotic embryo development. Thus you will learn not only about what methods can be used to visualize PCD in a multicellular organism and how these methods work, but also how embryo development proceeds in plants.

At early developmental stages, plant embryos are composed of two distinct parts. One part, called **embryonal mass**, establishes rudiments of all major tissues and organs and ultimately develops to plant. Another part, called **suspensor**, is composed of **terminally-differentiated cells** (these cells are unable to divide) that function as conduits of growth factors to the embryonal mass until the latter can develop autonomously. Thereafter the whole suspensor is eliminated. Terminal differentiation and subsequent elimination of suspensor cells are genetically controlled processes representing the earliest manifestation of PCD in plant development.

PCD is crucial for elimination of specific cells during development of an organism. In this practical somatic embryogenesis will be used as a model system to observe PCD. Somatic embryo development is initiated when the growth hormones cytokinin and auxin are removed from the cell culture. During this process ”suspensor

cells"[1] are progressively eliminated through PCD. The nuclei in these cells will be our target during the lab. You can find more about PCD in plant embryogenesis and about Norway spruce embryos, as a model system, at http://vaxt.vbsg.slu.se/~peterb/.

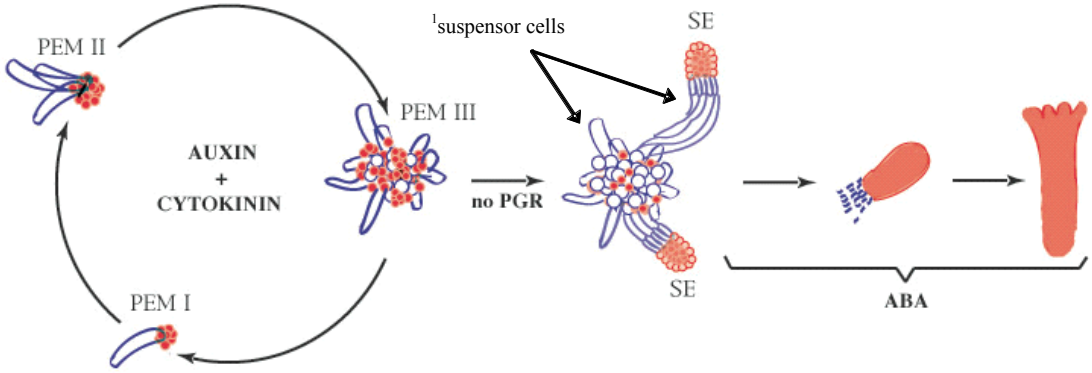


Figure 1. A schematic representation of the developmental pathway of somatic embryogenesis in Norway spruce (adapted from Filonova *et al*., 2000). Proliferation of Pro Embryonic Masses (PEMs) is stimulated by the hormones auxin and cytokinin. An individual PEM should pass through a series of three characteristic stages (I, II and III) to transdifferentiate to somatic embryos (SE). Withdrawal of plant growth regulators (PGRs) triggers embryo formation from PEM III, whereas the hormone abscisic acid (ABA) is necessary to promote further development of somatic embryos. Shown as dashed blue lines in the last but one stage of the pathway are the remnants of degenerated suspensor in beginning of late embryogeny (Filonova *et al*., 2000).

Methods for detection of PCD in spruce embryos

You will gain a further understanding of several methods for *in situ* detection of PCD. One method detects **nuclear DNA fragmentation**, which is a biochemical hallmark of PCD in diverse eukaryotic organisms, including plants. The DNA cleavage may yield double-stranded and single-stranded DNA breaks (nicks). Both types of breaks can be detected by labelling the free 3'-OH termini with modified nucleotides (*e.g.,* biotin-dUTP, DIG-dUTP, fluorescein-dUTP) in an enzymatic reaction. The enzyme **terminal deoxynucleotidyl transferase** (**TdT**) catalyzes the template-independent polymerization of deoxyribonucleotides to the 3'-end of single- and double-stranded DNA (Figure 2). This method is called **TUNEL** (**T**dT-mediated d**U**TP **n**ick **e**nd **l**abeling).

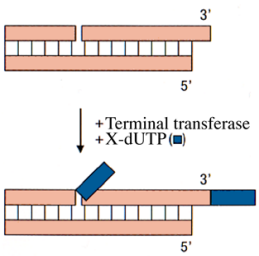


Figure 2. The general principle of TUNEL – template-independent labelling of single- and double-strand DNA breaks.

For TUNEL, you can use the *In Situ* Cell Death Detection Kit, TMR red (Roche). This kit contains two components: (i) TdT (Enzyme Solution) and (ii) tetramethylrhodamine (TMR)-conjugated dUTP (Label

Solution). Biological specimen (embryos in your case) are fixed and permeabilized. Subsequently, they are incubated with the **TUNEL reaction mixture** that contains TdT and TMR-dUTP. During this incubation period, TdT catalyzes the addition of TMR-dUTP at free 3'-OH groups in single- and double-stranded DNA. After washing, the label incorporated at the damaged sites of the DNA is visualized by flow cytometry and/or fluorescence microscopy. Since TUNEL-negative (*i.e.* living) cells do not emit any fluorescence and cannot be seen and/or counted, it is impossible to quantify the fraction of TUNEL-positive cells in the specimen without general nuclear staining. Therefore TUNEL-stained specimens are usually counterstained with a DNA fluorochrome. You can use 4,6-diamidino-2-phenylindole (**DAPI)**, as a counterstain. Slides can be viewed under a fluorescence microscope (Zeiss or Nikon) **in the dark room** (Figure 3)**. NOTE! Avoid exposing microscopic slides to direct light.** Excitation at 364 nm induces DAPI fluorescence (blue emission), while excitation at 543 nm induces TUNEL TMR-red fluorescence (red emission). Always view embryos under DAPI filter first and then change for TUNEL filter. Start with low magnification (x 20 objective) and then switch to a higher magnification (x 40 or x 60 objectives).

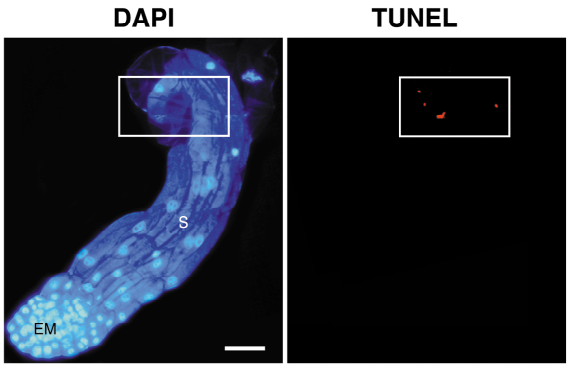


Fig. 3: TUNEL-positive nuclei are usually found at the distal part of the suspensor and are weaker stained with DAPI, as compared to TUNEL-negative nuclei.

Another method to detect PCD is to use the **Evan's blue** dye (an isomer of Trypan blue, which is widely used for testing viability of animal cells). In this case a second hallmark of PCD is explored and that is **damage/leakage of the plasma membranes.** Evan's blue is normally unable to penetrate the cells with intact plasma membrane due to its large molecular weight (MW 960), but is readily taken up by the cells undergoing cell death. This method is much less time-consuming as compared to other cell death assays including TUNEL. Cells are stained, washed to get rid of unbound dye and observed under light microscope. One should however remember that this method does NOT allow discriminating between necrosis and PCD, as both types of cell death exhibit permeability of the plasma membrane at certain stage of the cellular degradation. Thus Evan's blue staining should always be accompanied by other cell-death methods (*e.g.* TUNEL) to validate the occurrence of PCD. Figure 4 shows the general staining pattern of Norway spruce embryos with Evan's blue.

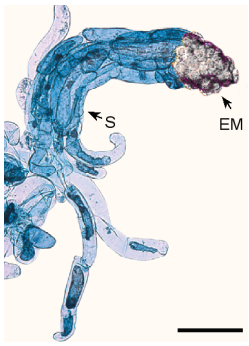


Figure 4: Evan's blue stained embryos usually reveal staining over the whole length of the suspensor and a lack of staining in the embryonal mass.
EM, embryonal mass
S, suspensor

Large molecular weight DNA binding dyes, such as propidium iodide (PI) or Evan's blue, cannot enter intact cells because of their large size. On the other hand, smaller dyes, such as DAPI, Hoechst 33342 or 33258, or calceinAM, can label both dying cells and living cells. As chromatin breaks down during PCD, the intensity of nuclear staining decreases, and this can be used as an assay of PCD progression. When the cell finally lyses and its membrane becomes permeable, then the larger-sized dyes (such as PI) can stain any DNA left within the cell. The altered morphology of nuclei selectively labelled with DNA-specific fluorescent dyes, such as DAPI or Hoechst dyes, can be a very easily interpreted assay for PCD (Willingham, 1999).

Overview of experimental procedure

The lab practical runs for half a day and you will stain a fresh batch of spruce embryos with PI and DAPI and analyse the stained embryos under light microscope and fluorescence microscope with simultaneous recording of your observations. The lab-supervisors will shortly describe how to perform TUNEL assay and Evan's blue staining, how to detect TUNEL-positive cells in the embryos in the fluorescence microscope, how to estimate the frequency of cells with DNA fragmentation, and how to compare nuclear morphology in the viable cells and those undergoing PCD.

Materials

Plant material

Embryos of Norway spruce (*Picea abies* L. Karst)

Chemicals

1µg/ml DAPI

1 mM PI (Propidium Iodine)

1x Phosphate buffered saline (PBS)

LP ½ medium

Accessories

Pipettes 1000 µl, 20 µl	Falcon tubes 15 ml	Eppendorf tubes 2 ml
Pasteur pipettes	Polysine microscope slides	Pens
Cover slips		

Microscopy

Light microscope

Microscope with Ultraviolet light *e.g.* the Ziess microscope (including DAPI and PI filter, *i.e.* BLUE and RED emission filters)

Procedures

Wear gloves!

Three experiments will be prepared (i) control, (ii) DAPI and (iii) DAPI + PI. For each preparation approximately 0.3 ml cells are enough.

1. Use three 2 ml Eppendorf- tubes, mark these according to the experiments above i, ii and iii.
2. Suck up 2 ml cell suspension (0.3 ml cells + 1.7 ml media) to each of the Eppendorf-tubes. If solid cell culture cut out a piece of cells/embryos and "dissolve" in approximately 2 ml of culture media.
3. First let the cells settle at the bottom of the tube, at least 0.3 ml cells, remove all supernatant.
4. Add 2 ml DAPI-stain solution to tubes (ii) and (iii).
5. Add 6 µl of 1 mM PI stock to tube (iii) (final concentration of PI is 30 µM).
6. Incubate all three tubes for 15 min at room temperature, gently flip tubes during incubation.
7. Let the cells settle, collect supernatant and transfer to falcon tubes for disposal to chemical waste. Wash the cells two times with PBS (phosphate buffer) pH 7.4. Leave the PBS in the last wash.
8. Divide the cell suspension on several object slides, cover with cover slips
9. First, use light microscopy to distinguish between embryonal mass and suspensor cells). Then use UV-light and specific filter to visualise the blue DAPI and the red PI fluorescence. Try to find the differences in fluorescence intensity of DAPI between intact nuclei in living cells and degraded nuclei in dying cells.

Tip: compare embryonal mass (compact cells) and the vacuolated suspensor cells.

Lab report

1. Follow the general instructions on how to write a report.
2. Give the tasks and brief description of the model system and methods and any deviations from the lab manual.
3. Document your results.
4. Answer following questions:
    - What happens to nuclei and nuclear DNA during PCD and which mechanisms cause nuclear degradation?
    - What happens to plasma membrane during PCD and what are the consequences of these changes?

- Give examples of other techniques for analysis of DNA fragmentation. What are the advantages and disadvantages of TUNEL over other techniques?
- Why will not all Evan's blue or PI positive cells in the Norway spruce embryos be positive for TUNEL?
- Some embryos can contain TUNEL, Evan's blue and/or PI positive cells in the embryonal masses. What does this tell you?
- What are the morphological differences between cells in the embryonal masses and in the suspensors?
- How would Evan's blue or PI staining pattern change if embryos were pre-treated with hydrogen peroxide?
- How would Evan's blue or PI staining pattern change if suspensor cells could avoid terminal differentiation and start dividing?

References

Filonova; L.H. et al.(2000). Two waves of programmed cell death occur during formation and development of somatic embryos in the gymnosperm, Norway spruce. *J Cell Sci* 113, 4399-4411.

Willingham M.C. (1999). Cytochemical Methods for the Detection of Apoptosis. *J. Histochem. & Cytochem.* 47(9):1101–1109

Risks and protection

Chemicals:
- Potassium cacodylate and cobalt dichloride in TUNEL Label Solution – potential carcinogens - gloves
- DAPI – skin irritant - gloves

Organisms:
- Not applicable

Radioactivity:
- Not applicable

Other:
-

Risks and Protection

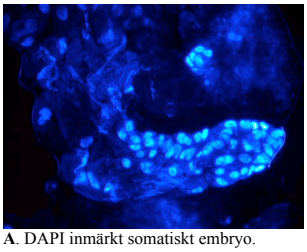
Chemicals:
DAPI (4',6-Diamidino-2-phenylindole dihydrochloride)
Biochem/physiol Actions
Cell permeable fluorescent minor groove-binding probe for DNA. Binds to the minor groove of double-stranded DNA (preferentially to AT-rich DNA), forming a stable complex which fluoresces approximately 20 times greater than DAPI alone.
Application
DAPI is several times more sensitive than ethidium bromide for staining DNA in agarose gels. It may be used for photofootprinting of DNA, to detect annealed probes in blotting applications by specifically visualizing the double-stranded complex, and to study the changes in DNA and analyze DNA content during apoptosis using flow cytometry. DAPI staining has also been shown to be a sensitive and specific detection method for mycoplasma.
Fluorescence ex 364 nm;emm 454 nm (DAPI-DNA-complex)
NB! Carcinogenic binds irreversible to DNA

PI (Propidium Iodine) Stains Nucleic acids
Application
Propidium iodide has been widely used as a fluorescent stain for DNA in fixed cells, sometimes in conjunction with fluorescamine to give information concerning protein, DNA and protein-to-DNA distribution. 4-8 It has been used as a fluorescent probe for acetylcholinesterase (site and specificity of binding). Propidium iodide binds to double-stranded DNA. Propidium iodide readily enters and stains non-viable cells, but cannot cross the membrane of viable cells. Its fluorescence above 630 nm allows its use as an indicator of cellular DNA content and to be used with fluorescein or phycoerythrin in immunofluorescent cell viability screening.
Fluorescence ex 493 nm;emm 630nm (PI-DNA-Komplex)
NB! Carcinogenic binds irreversible to DNA

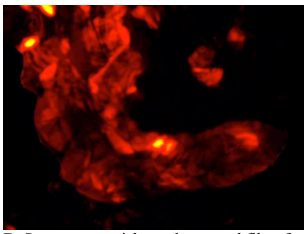
UV-light, it will give permanent damage to your eyes if they are exposed to the light source.

Appendix 1

Examples of Propidium Iodine (PI) and DAPI stained somatic spruce embryos from previous courses

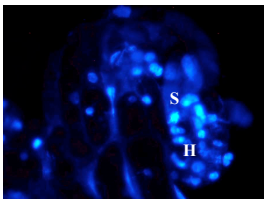


A. DAPI inmärkt somatiskt embryo.

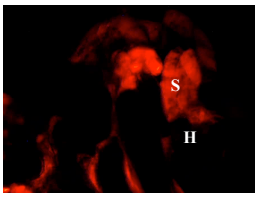


B. Samma somatiska embryo med filter för PI. Propidium Iodide är en stor florocerande molekyl som fäster till DNA. Eftersom den är så stor kommer den inte kunna passera genom ett cellmembran i intakta celler. Därför syns bara cellkärnor i döda celler. Dessa är framförallt lokaliserade i ES. PI lyser med ett rött sken.

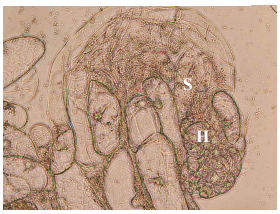
Figure 1. One somatic spruce embryo labelled with DAPI (A) and PI (B). These pictures come from a previous course, and the experiment was preformed by Katarina Lyberg and Louise Lund.



A: DAPI staining of an embryo. S: Suspensor; H: Head



B: PI staining of an embryo. S: Suspensor; H: Head



C: The embryo under light microscope (same embryo as in A and B)

Figure 2. One somatic spruce embryo labelled with DAPI (A) and PI (B) and in light microscope. These pictures come from a previous course, and the experiment was preformed by Jing Huang and Lisa Schuster.