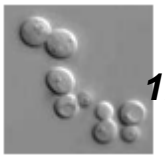
Materials and Methods



1.1. Yeast methods

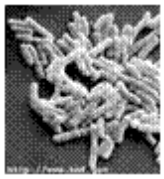
1.1.1. LiAc Yeast Transformation

Solutions:

- Synthetic drop out solution 10 x in AD: L-isoleucine 300 mg/L, L-valine 1.5 g/L, L-adenine hemisulfate salt 200 mg/L, L-arginine HCl 200 mg/L, L-histidin HCl monohydrate 200 mg/L, L-leucine 1 g/L, L-lysine HCl 300 mg/L, L-methionine 200 mg/L, L-phenylalanine 500 mg/L, L-threonine 2 g/L, L-tryptophan 200, L-tyrosine 300 mg/L, L-uracil 200 mg/L
- SD -Trp medium (synthetic dropout medium): synthetic minimal medium lacking tryptophan: yeast nitrogen base without amino acids 6.7 g/L, 2 % dextrose (glucose) (sterile dextrose solution is added after autoclaving to avoid maillard reactions), pH adjusted to 5.8, for plates : agar 1.5 g/L
- YPD (yeast peptone dextrose) broth, yeast complete medium: yeast extract 10 g/L, peptone 20 g/L, 2 % dextrose (glucose), pH adjusted to 5.8
- Aqua dest. sterile
- LiAc 100 mM sterile
- LiAc 1 M sterile
- Bacterial RNA, used as carrier
- Poly-ethyleneglycol PEG 50 % (w/v) sterile filtered

10 mL of SD -Trp medium are inoculated with the appropriate yeast strain and incubated at 30°C while shaking at 200 rpm o/n. On the next day OD at 600 nm is measured and the yeast culture is diluted with YPD to OD_{600} 0.1. A total volume of 50 mL diluted yeast culture is used for further incubation. Every hour OD_{600} is measured until OD_{600} 0.4 is reached (3 - 5 hours). Then the cell number is calculated with a Thoma chamber. $2x10^7$ cells/mL are sufficient for 10 transformations. The yeast is then harvested by centrifugation at 3000 rpm for 5 minutes, the supernatant is carefully removed and collected for autoclaving. The pellet is resuspended in 25 mL sterile AD and again centrifuged at 3000 rpm for 5 minutes. After removing of the supernatant the pellet is resuspended in 1 mL LiAc 100 mM. Excess of LiAc is removed by spinning the tubes for 15 seconds at full speed in a tabletop centrifuge and carefully removing the supernatant. The yeast pellet is brought to a final volume of 500 µL with LiAc 100 mM. Aliquots of 50 µL are prepared. One 50 µL aliquot of this yeast suspension is used for one transformation. 50 µL aliquots are again briefly centrifuged to pellet the cells, the supernatant is removed and on top of the yeast pellet, layers of the following transformation solutions are pipetted in following order: 240 µL 50 % PEG, 36 µL LiAc 1 M, 3.3 µL of bacterial RNA (31 µg/µL), 70.7 µL sterile AD, 1 µg plasmid DNA (1µg/µL). The tube is then thoroughly mixed by vortexing for 1 minute until the yeast pellet is

completely dissolved and placed for 30 minutes in a 30°C water bath. The tube is then transferred to a 42°C water bath for 25 minutes in order to perform the heatshock. The transformation mix is then briefly centrifuged for 15 seconds at 4 000 x g (7 000 rpm) in a table top centrifuge, the supernatant is discarded and the pellet is resuspended in 1 mL sterile AD. 50 µL of this transformed yeast suspension are plated on SD – Leu, - Trp, - Ade plates and incubated at 30°C for some days.



1.2. Bacteria and DNA Methods

1.2.1. E. coli over night Culture

Solutions:

- LB (Luria bretani) broth: 10 g tryptone, 5 g yeast extract, 10 g NaCl, in 1 L AD, pH 7.0. Sterilization by autoclaving
- Antibiotic stock solutions 1000x: ampicillin 100 mg/mL, kanamycin 25 mg/mL

For preparing a saturated E. coli over night culture, 5 mL LB broth containing antibiotics are inoculated with a single colony of the desired bacteria strain in a 50 mL tube. Incubation is carried out at 37°C with constant shaking at 200 rpm over night (o/n). After 12 hours the culture is in the state of stationary phase, no more increase of biomass is observed.

1.2.2. Preparation of Electro Competent E. coli

Solutions:

- LB broth
- AD sterile, 4°C
- 10 % glycerol, sterile filtered, 4°C
- liquid N_2

10 mL LB broth are inoculated with E. coli HB101 and an o/n culture are prepared. On the next day the o/n culture is diluted in 1 L LB broth and incubation is continued for around 3 hours. Optical density (OD) at 600 nm is measured after 1 hour respectively until OD 0.4 - 0.6 is reached. By that means the bacteria are in the state of exponential phase. The bacteria broth is then cooled down on ice for 15 – 30 minutes in order to stop the bacteria from dividing. Harvesting of bacteria is carried out by centrifugation at 2500 x g, 4°C for 15 minutes (centrifuge: Sorvall RC-5B), the supernatant is removed and collected for autoclaving. The bacteria pellet is resuspended in 1 L sterile AD (4°C) and again centrifuged

at 2500 x g for 15 minutes at 4°C. The supernatant is discarded and the pellet is resuspended in 500 mL sterile AD (4°C) and again centrifuged at 2500 x g for 15 minutes at 4°C. The supernatant is discarded and the pellet is resupended in 20 mL 10 % sterile glycerol (4°C) and again centrifuged at 2500 x g for 15 minutes at 4°C. The supernatant is discarded and the pellet is resuspended in 2 - 5 mL 10 % sterile glycerol (4°C). 50 µL aliquots of this bacteria solution are prepared and quickly frozen in liquid N_2. Aliquots are stored at -80°C and can be used for 1 year.

1.2.3. Electroporation Transformation of E. coli

Solutions:

- SOC medium for recovery: LB broth containing glucose 20 mM, $MgSO_4$ 10 mM, $MgCl_2$ 10 mM

50 µL electro-competent bacteria aliquot is thawed on ice and transferred into prechilled electroporation cuvettes, 0.5 - 1 µg plasmid DNA (or 5 - 10 µL of ligation mix) is added (0.1 cm). The surface of the cuvette was dried prior to electroporation. Electroporation parameters (power supply : voltage: 1.25 – 1.9 kV, capacity: 25 µF, resistance: 200 Ω. A successful electroporation is achieved with a time constant between 4 – 5 ms. After electroporation the bacteria are resuspended in 1 mL of SOC medium and transferred in a 3059 Falcon tube. The bacteria are then incubated at 37°C at 200 rpm for 1 hour. After recovery the transformed bacteria are briefly centrifuged, resuspended in 100 µL SOC medium and the suspension is plated on LB agar plates containing appropriate antibiotics. The plates are incubated over night at 37°C.

1.2.4. Preparation of Heat Shock Competent E. coli

Solutions:

- LB broth
- TSS Buffer: 1 % Bacto-Trypton, 0.5 % yeast extract, 0.5 % NaCl, 5 % DMSO (dimethylsulfoxide), $MgCl_2$ 50 mM, pH 6.5, 10% PEG (MW 3000, 3350), fill up with AD, sterile filtered, stored at 4°C.
- Glycerol 87 %
- Liquid N_2

10 mL LB broth are inoculated with E. coli HB101 or another appropriate strain such as DH5α and an o/n culture is prepared. On the next day the o/n culture is diluted in 1 L LB broth and incubation is continued for around 3 hours. Optical density (OD) at 600 nm is measured after 1 hour respectively until OD 0.4 – 0.6 is reached indicating that the bacteria are in the exponential phase of growth. The bacteria broth is then cooled down on ice for 15 – 30 minutes in order to stop the bacteria from dividing. Harvesting of bacteria is carried out

by centrifugation at 2500 x g, 4°C for 15 minutes (centrifuge: Sorvall RC-5B). The supernatant is removed and collected for autoclaving. The pellet is resuspended in a total volume of ~ 50 mL (36 mL TSS buffer + 12 mL of glycerol, 1/20 volume of diluted culture). Aliquots of 200 µL are prepared, quickly frozen in liquid N_2 and stored at -80°C.

1.2.5. Heat Shock Transformation of E. coli

Solutions:

- SOC medium: LB broth containing 0.36% glucose, $MgSO_4$ 10 mM, $MgCl_2$ 10 mM

An aliquot of 50 µL heat shock competent E. coli (DH5α, stored at –80°C) is quickly thawed in the palm and left for 10 minutes on ice. The aliquot is then transferred into a pre-chilled Falcon 2059 tube. About 1 µg of DNA (1 - 2.5 µL) is pipetted into the competent bacteria and the tube is gently swirled. After 20 minutes of incubation on ice the tube is placed in a prewarmed 42°C water bath for 90 seconds without moving the tube. In order to cool down the sample, the tube is immediately placed on ice for 1-2 minutes. Thereafter 800 µL of SOC medium are added to the transformation mix and the bacteria are incubated for 1 hour at 37°C and 200 rpm to allow recovery from the heat shock and start expression of the selection gene. Plating: For simple retransformations 100 µL are plated on appropriate LB (Luria Bertani) agar plates (prewarmed to 37°C) supplemented with the appropriate antibiotics (kanamycin 25 µg/mL or ampicillin 100 µg/mL). For clonings after a ligation the whole bacterial suspension is used by pelleting the bacteria of the transformation mix by briefly and gently spinning down, discarding the supernatant and resupension of the pellet in 100 µL SOC medium (or the reminder of the supernatant). This concentrated suspension containing all the bacteria is then plated on LB agar plates containing appropriate antibiotics. The plates are incubated over night at 37°C.

1.2.6. Glycerol Stock of E. coli

Solutions:

- Glycerol 87 %

For a -80°C E. coli glycerol stock, 400 µL of an E. coli o/n culture are mixed thoroughly with 100 µL 87 % glycerol in a 1.5 ml reaction tube and stored at -80°C. For inoculation of fresh LB broth, a yellow 200 µL tip is plunged into the frozen (!) stock and then pipetted up and down in the LB broth in order to thaw and resuspend the bacteria.

1.2.7. Crude Plasmid DNA Preparation by Alkaline Lysis

Solutions: Qiagen Plasmid Preparation Kit

- Resuspension buffer (P1): Tris/HCl 50 mM pH 8.0, EDTA 10 mM, RNase A 100 µg/mL , stored at 4°C

- Lysis buffer (P2): NaOH 200 mM, 1 % SDS
- Neutralization buffer (P3): KAc 3 M pH 5.5
- Isopropanol, room temperature
- Ethanol 70 %, 4°C

50 mL Falcon tubes filled with 5 mL of LB broth containing appropriate selective antibiotics (ampicillin: 100 µg/mL or kanamycin: 25 µg/mL) are inoculated with one colony of the desired transformed E. coli strain and incubated o/n at 37°C with vigorous shaking at 200 rpm. On the next day, a glycerol stock is made and then the cells are harvested with centrifugation at 6000 rpm? for 15 minutes at 4°C (centrifuge: Heraeus model Biofuge Primo R), the supernatant is removed and collected for autoclaving. Leftovers of LB broth can decrease the yield of plasmid DNA, therefore the Falcon tubes are placed upside down on a paper towel for some minutes in order to remove as much LB broth as possible. The E. coli pellet is resuspended in 250 µL of Resuspension buffer, transferred to 1.5 mL reaction tubes, then 250 µL of Lysis buffer are added and the lysis mix is carefully inverted 5 – 6 times until the solution is viscous. Shearing of genomic DNA by heavy shaking should be avoided. After 5 minutes incubation time at room temperature 250 µL of Neutralization buffer are added and again the mix is carefully inverted several times until a white precipitate of genomic DNA and proteins is visible. The white precipitate is pelleted by centrifuging at 6000 g for 15 minutes at 4°C. The supernatant containing plasmid DNA is collected without perturbing the pellet and centrifuged a second time to quantitatively remove genomic DNA precipitate. The collected supernatant is then subjected to isopropanol precipitation. The unpurified plasmid DNA solution is mixed with 0.7 volumes of isopropanol. After 5 minutes of incubation at room temperature, precipitated plasmid DNA is recovered with centrifugation at full speed, 16 000 x g, at 4°C for 30 minutes (centrifuge: Heraeus Sepatech Megafuge 1.0 R), the supernatant is removed and discarded. The plasmid DNA pellet is washed with 1 mL 70 % EtOH (4°C) without disturbing the pellet and centrifuged for another 5 minutes at full speed at 4°C and the supernatant is quantitatively removed. The pellet is dried for 5 min at RT and dissolved in 20 µl AD or 1x TE buffer. In some cases this DNA-solution was further purified by an EtOH/NaAc precipitation. The crude preparation of plasmid DNA can be subjected to restriction digest analysis and sequencing but cannot be used for transfection experiments of mammalian cells. In the latter case, the crude DNA solution was purified by Qiagen DNA purification columns.

1.2.8. Ethanol/NaAc Precipitation of Plasmid DNA

Solutions:

- Ethanol 70 %, -20°C
- Ethanol 70 %, 4°C
- NaAc 3 M
- TE buffer: Tris/HCl 10 mM pH 7.5, EDTA 1 mM

The DNA solution or in some cases the restriction digest sample was mixed with 1/10 volume of NaAc, 2.5 volumes of 100 % ethanol (-20°C) and was quickly transferred to -70°C for 5 minutes incubation. Then the plasmid was collected by centrifugation for 30 minutes at full speed, 16 000 x g, 4°C (centrifuge: Heraeus Sepatech Megafuge 1.0 R), the supernatant was removed by suction. The pellet was washed with 1 mL 70 % ethanol (4°C) and again centrifuged for 5 minutes at 14 000 rpm, 4°C and the supernatant was quantitatively removed by suction. The pellet was air-dried for some minutes prior to elution in an appropriate volume of AD or 1x TE buffer (to reach a final concentration of about 1 µg/µl).

1.2.9. Plasmid DNA Preparation by Alkaline Lysis:

Solutions:

- Equilibration buffer (QBT): NaCl 750 mM, MOPS 50 mM pH 7.0, 15 % isopropanol, 0.15 % Triton X-100, stored at room temperature
- Wash buffer (QC): NaCl 1 M, MOPS 50 mM pH 7.0, 15 % isopropanol, stored at room temperature
- Elution buffer (QF): NaCl 1.25 M, Tris/HCl pH 8.5, 15 % isopropanol, stored at room temperature

For midi (maxi) DNA preparations the QIAGEN Plasmid Midi (Maxi) kit was used (Cat.no.12143, 12163). A 250 mL (500 mL) shaking flask filled with 50 mL (100 mL) LB broth containing appropriate selective antibiotics (ampicillin 100 µg/mL, kanamycin 25 µg/mL) were inoculated with the desired transformed single colony of E. coli strain and incubated o/n at 37°C with vigorous shaking at 200 rpm. The cells were harvested by centrifugation at 6000 g for 15 minutes at 4°C (centrifuge: Heraeus model Biofuge Primo R), the supernatant was removed and collected for autoclaving. Leftovers of LB broth can decrease the yield of plasmid DNA, therefore the centrifugation buckets were placed upside down on a paper towel for some minutes in order to remove as much LB broth as possible – remnants of LB on the walls of the tubes were removed by suction. The E. coli pellet was resuspended in 4 mL (10 mL) of Resuspension buffer P1, 4 mL (10 mL) of Lysis buffer (P2) were added and the lysis mix was carefully inverted 5 – 6 times. After 5 minutes incubation time at room temperature 4 mL (10 mL) of Neutralization buffer were added, again the mix was carefully inverted several times until a white precipitate of genomic DNA and proteins were visible and the neutralized lysate was incubated on ice for 20 minutes. The white precipitate was pelleted by centrifuging at 6000 g for 15 minutes at 4°C. The supernatant containing plasmid DNA was collected without disturbing the pellet and centrifuged a second time 6000 g for 15 minutes at 4°C. In the mean time a Quiagen tip 100 (500) was equilibrated by applying 4 mL (10 mL) of Equilibration buffer on the matrix of the tip, the buffer entered the resin only by gravity flow. The supernatant of the centrifugation was applied on the columns and entered the column by gravity flow. 2 Washing procedures were performed with 10 mL (30 mL) of Wash buffer respectively. Purified plasmid DNA was eluted with 5 mL (15 mL) Elution buffer. After elution DNA was precipitated with 0.7 volumes

isopropanol, pelleted by centrifugation at 14 000 rpm for 30 min at 4°C centrifuge: Sorvall RC-5B rotor SA 600), the pellet was air-dried and eluted in 100 µL (500 µl) AD or 1x TE. DNA yield was quantified with an UV spectrophotometer and the plasmid DNA was diluted to a final concentration of 1 µg/µL.

1.2.10. Isopropanol Precipitation of Plasmid DNA

Solutions:

- Isopropanol, room temperature
- Ethanol 70 %, room temperature

The eluted midi (maxi) plasmid DNA solution was mixed with 0.7 volumes of isopropanol (room temperature) and immediately centrifuged for 30 minutes at full speed, 17 000 x g, 4°C (centrifuge: Heraeus Sepatech Megafuge 1.0 R). The supernatant is carefully removed. The DNA pellet is washed with 2 mL (5 mL) 70 % ethanol (room temperature) without disturbing the pellet and another centrifugation step is carried out for 10 minutes at full speed, 4°C, the supernatant is quantitatively removed by suction. The pellet is air dried and dissolved in 100 µL AD. DNA yield is quantified and the plasmid DNA is diluted to a final working concentration of 1 µg/µL.

1.2.11. DNA Quantification

DNA exhibits a specific absorption maximum at 260 nm, which is used for DNA quantification by an UV/VIS spectrophotometer (Pharmacia Biotech Ultrospec 2000). An OD at 260 nm of 1 refers to a DNA amount of 50 µg/mL. An appropriate dilution (1:200 after midi or maxi prep) of the DNA sample is necessary to stay in the linear range (0.1-1) of this quantification method. The purity of DNA concerning the presence of residual proteins can be calculated by the ratio of OD260/OD280. The aromatic amino acid tryptophan exhibits an absorption maximum at 280 nm, which refers to the relative amount of protein in the sample. An OD260/OD280 ratio of 1.8 – 2.0 is desired – which is typical for pure DNA samples resulting from the shoulder of DNA-absorbance at 280 nm.

1.2.12. Polymerase Chain Reaction – PCR

Solutions:

- AD nuclease free
- dNTP mix, each nucleotide 2 mM: stock solutions 100 mM
- Forward and reverse primers 10 pmol/µL in AD respectively
- 10x Pfu puffer (or Taq buffer)
- Template DNA
- Pfu polymerase 2.5 U/µL – or Taq polymerase 2.5 U/µL (Pfu is used for cloning purposes due to the higher fidelity of this DNA polymerase; Taq polymerase is used

for analytical PCR's)

For a 100 µL PCR reaction, following solutions are mixed in a thin walled reaction tube (BioRad): 85 µL AD, 10 µL Pfu buffer 10x, 1 µL dNTP mix 2 mM, 1 µL forward primer 10 pmol/µL, 1 µL reverse primer 10 pmol/µL, 500 ng (1 µL) template DNA, 1 µL Pfu polymerase (2.5 U). Handling and pipetting of PCR steps are carried out with gloves in order to prevent contaminations. The reaction tubes arere placed in the hot (94°) PCR block (Perkin Elmer Gene Amp PCR System 2400) and the following program is used:

step 1 10 minutes 94°C (denaturation)
step 2 1 minute 94°C (denaturation)
step 3 1 minute 56°C or other appropriate annealing temperature – about 2-4 °C below the calculated melting temperature (annealing)
step 4 1.5 minutes/ 1kb 72°C (elongation)
step 5 5 minutes 68°C
step 6 ∞ 4°C (cooling)

30 cycles of steps 2-4 are carried out. After finished PCR, 6x DNA sample buffer is added to the PCR samples to inactivate the polymerase. Amplified PCR products are resolved by 1 % agarose gel electrophoresis.

1.2.13. Single Restriction Digest

Restriction digest of 20 µL volume: 17 µL of Aqua dest. (nuclease free) are mixed with 2 µL appropriate 10x restriction enzyme buffer, 1 µg (1µg/µL) of DNA added and mixed well. Then 1 µL (10 U) of restriction enzyme (-20°C) are added and again mixed well by swirling and spinning down. After incubation time of 1 hour at 37°C in a water bath (or other appropriate temperature according to manufacturers protocol) DNA fragments can be resolved by agarose gel electrophoresis.

1.2.14. Ligation

Solutions:

- T4 ligase buffer 10x (New England Biolabs, cat. no. #202S)
- T4 ligase 400 u/µL (New England Biolabs, cat. no. #202S)
- AD nuclease free

For a ligation reaction of total 10 µL, the following solutions are mixed: 100 – 200 ng linearized purified vector DNA, an equimolar or slightly higher amount of purified insert DNA, 1 µL T4 ligase buffer 10x, 0.5 µL T4 ligase and AD to a total of 10 µl. As a ligation control, another ligation reaction missing the insert DNA is prepared, instead of the insert DNA the equivalent volume of AD is added to the solutions. The ligations are either

incubated at 16°C over night or at room temperature for 4 hours. 5 µL of each ligation sample are used for heat shock transformation or electroporation.

1.2.15. Agarose Gel Electrophoresis

Solutions:

- TAE 50x stock: Tris base 2 M, 57.1 mL acetic acid, EDTA 50 mM (stock 1 M pH 8), ad 1 L with AD
- Ethidiumbromide 10 mg/mL in aqua dest.
- Running buffer: TAE 1x
- DNA sample buffer 6x:
  (Fermentas:10mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60mM EDTA

Typically, 1 % agarose gels are poured by heating up 1 g agarose / 100 mL 1 x TAE until agarose is completely dissolved and no smears are visible. The hot agarose solution is cooled down to about 55°C and 5 µL ethidium bromide solution are added and gently mixed to avoid formation of bubbles. Then, the agarose solution is poured immediately into the gel cast cassette, appropriate combs are placed in the solution and abundant bubbles are pushed away from the slots by pipette tips. After about 30 minutes, polymerization is finished and the agarose gel can be used for electrophoresis. The agarose gel is placed in the electrophoresis chamber (BioRad) with the slots facing the cathode and 1x TAE is added until the gel is sufficiently covered by running buffer. If necessary, the slots are rinsed with running buffer by pipetting up and down some running buffer in order to remove leftovers of agarose slurry. DNA samples are mixed with 6x DNA sample buffer (5:1) and loaded in the slots. After loading, electrophoresis is started immediately in order to prevent diffusion of sample into the surrounding gel. Electrophoresis parameters (power supply BioRad Power PAC 2000): 100 – 120 V for at least 30 minutes depending on the DNA fragment size supposed being resolved. Resolved DNA fragments are visualized under UV transillumination (transilluminator: 260 – 340 nm range) and documented with a BioRad gel doc system. For clonings care is taken that the DNA is not exposed too long to UV light since UV light can irreversibly damage DNA. DNA fragments are excised with a clean razorblade and gel extraction is carried out as described below.

1.2.16. Gel Extraction

Solutions:

- Solubilization buffer (QG)
- Isopropanol
- Wash buffer (PE)
- Elution buffer (EB): Tris/HCl 10 mM pH 8.5

For gel extractions usually the QIAquick gel extraction kit is used (Qiagen, Cat.no. 28704). An excised agarose gel slice is weighed to calculate the approximate gel slice volume (1 g = 1 mL) and transferred into an reaction tube. 3 volumes of solubilization buffer are added and incubated in a 50°C waterbath until the gel piece is completely dissolved. 1 gel volume isopropanol is added and mixed. The solution is then loaded on the column and centrifuged for 1 minute at full speed (centrifuge: Eppendorf centrifuge 5415 D), the flow through is discarded. 750 µL of wash buffer are pipetted to the column and centrifuged under the same conditions as above, the flow through is discarded and the column is placed in a new microreaction tube. For elution of extracted DNA, 20 µL of elution buffer are directly applied on the matrix, incubated for 1 minute at room temperature and centrifugation as above is carried out. As an alternative to the Qiagen gel extraction kit, sometimes GenElute columns (Sigma, cat. no. # 5-6500) are used, which are equilibrated by loading of 100 µl 1x TE buffer, followed by brief centrifugation (1 min at full speed), addition of the gel slice to the column and centrifugation at full speed for 10 min. The resulting flow through (about 40 µl) contains about 90 % of the DNA, while the agarose remains in the column.

1.3. Mammalian Cell Culture Methods

1.3.1. Cell culture of 293, HeLa, MEF, stable transfected 293 Cell Lines

Solutions:

- Dulbecco's modified Eagle's medium complete (DMEM complete) containing 10 % FCS (fetal calf serum), penicillin (100 u/mL), streptomycin (100 µg/mL), glutamine 2 mM (stock solutions of penicillin, streptomycin and glutamine: 100x)
- DMEM G418 medium: DMEM complete containing G418 500 µg/mL
- G418 stock solution: 500 mg/mL G418 in Hepes 100 mM
- PBS deficient: NaCl 8 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g, with AD to 1 L, pH adjustment with HCl to pH 7.4 (provided by Novartis facility)
- Trypsin solution 0.25 % in Hepes 10 mM

293 cells (immortalized human embryonic kidney cells, adherent), HeLa (human cervix carcinoma cells, adherent) and MEF (mouse embryonic fibroblasts, adherent) are cultured in DMEM complete medium, in addition to this DMEM complete medium, the medium for stable 293 cell lines with a genomic integration of a neomycin-resistance plasmid containing the gene of interest, contains 500 µg/mL G418 as permanent selection agent. Cells are cultivated in 75 cm^2 tissue culture flasks at 37°C, in a humidified (90 %) and CO_2 (5 %) containing atmosphere. All cell types are trypsinized and passaged every second or third day in proportion of 1:4 or 1:6, respectively. For passaging the cell culture medium is removed, about 13 mL of PBS def. (room temperature) are used to wash the cells. PBS def. is removed as well and about 3 mL trypsin solution (37°C) are pipetted into the flask and

spread evenly all over the bottom. Excess of trypsin is removed and the flask is placed in the incubator for around 3 minutes. With an inverse microscope (Nikon TMS F 4x or 10x objective) it is checked if the cells are already detached and round in shape. Trypsinized cells are gently resuspended by several times up/down pipetting in 8 mL (to 12 ml – according to the desired passaging ratio) complete DMEM and 2 mL are transferred into a new tissue culture flask containing 13 mL of DMEM complete or DMEM complete including G418. Tissue culture flasks prepared for MEFs have to be gelatinized prior to passaging as follows: 15 mL of 1 % gelatin solution (autoclaved and sterile filtered, 37°C) are pipetted in a 75 cm^2 tissue culture flask and incubated at 37°C for 30 minutes, the gelatin solution is discarded afterwards. After this procedure, passaging is performed as usual.

1.3.2. Freezing of Mammalian Cell Lines

Solutions:

- Freeze medium: DMEM, 10 % (v/v) DMSO (dimethylsulfoxide), 20 % (v/v) FBS (fetal bovine serum), sterile filtered

293, HeLa, MEF, stable transfected 293 cell lines are passaged as described above, seeded in 75 cm^2 cell culture flasks and grown until 100 % confluency is reached. The cells are trypsinized as described above, resuspended in DMEM complete medium (4°) and centrifuged at 1000 rpm (400 g), 4°C for 5 minutes in a cell centrifuge with a swing-out rotor. The supernatant is removed and the cell pellet of one 75 cm^2 cell culture flask is resuspended in 2 mL freeze medium (4°C). 1 mL of resuspended cells are pipetted in pre-chilled cryotubes, placed in an isopropanol-box (“Mr Freeze”, 4°C) and then transferred to -80°C in order to freeze the cells in a controlled and slow procedure. After 24 hours the cell aliquots are transferred and stored in the liquid nitrogen tank at -196°C.

1.3.3. Thawing of Mammalian Cell Lines

A cell aliquot of 293, HeLa, MEF or stable transfected 293 cell lines stored in liquid nitrogen is quickly thawed in a 37°C water bath with constant moving to prevent local overheated spots warmer than 4°C. The thawed cell suspension is resuspended and diluted in about 20 mL DMEM complete medium and the cells are centrifuged at 1000 rpm for 5 minutes at 4°C. The supernatant is removed by suction, the cell pellet is resuspended in DMEM complete medium or supplemented with G418 and the cells are seeded in 75 cm^2 cell culture flasks.

1.3.4. $CaCl_2$ Transfection

Solutions:

- $CaCl_2$ 2 M in Aqua dest:
- HeBS (Hepes buffered saline): Hepes 50 mM, NaCl 280 mM, Na_2HPO_4 1.5 mM, pH adjustment to exactly pH 7.05 with HCl, sterile filtered (0.2 µm pore size), stored at -20°C

Preparation: 293 or HeLa cells are passaged and seeded at a density of 500.000 cells/6 well (40 % confluence). After one day (80 % confluence) transfection is carried out. Transformation mix: 2 µg of DNA (1 µg/µL) are diluted in 60 µL of aqua dest. in a polystyrene tube and 9 µL of 2 M $CaCl_2$ are added and mixed, 71 µL of HeBS are added dropwise by unscrewing a P200 Gilson Pipette while constantly vortexing at half speed. After incubation of 5 minutes at room temperature the solution is pipetted dropwise as evenly as possible on the surface of the cell culture dish. Once the DNA/calcium-precipitates get in contact with the cells the tissue culture dish should not be moved a lot. Normally the proteins of interest are expressed sufficiently after 24 hours of transfection (when CMV promoter containing plasmids are used).

1.3.5. Lipofectamine Transfection of Mammalian Cell lines

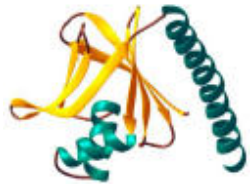
Solutions:

- Sterile DMEM medium without any additives (DMEM ∅)
- DMEM complete
- Lipofectamine Plus reagent
- Lipofectamine reagent

Preparation: 293 or HeLa cells are passaged and seeded at a density of 500,000 cells/6 well (40 % confluence). After one day (80 % confluence) transfection is carried out. For transformation of one 6-well 293 (HeLa) cells, 100 µL DMEM ∅ medium is mixed with 1 µg (1.5 µg) plasmid DNA and 4 µL (5 µL) lipofectamine Plus reagent and incubated for 15 minutes at room temperature. 100 µL of DMEM ∅ are mixed with 3 µL (3 µL) lipofectamine reagent added to the incubated solution and mixed gently. The lipofectamine/DNA solution is further incubated at room temperature for 15 min. Thereafter, the transformation mix is diluted with 800 µL DMEM ∅. The culture medium of 293 or HeLa cells is removed by suction, the cells are carefully washed once with at least 1.5 mL DMEM ∅ medium, the wash DMEM is removed and the transformation mix is carefully added to the cells. The 293 (or HeLa) cells are incubated with the transformation mix in the incubator for 3 hours (7 hours). After the transformation period the transformation medium is removed and replaced by DMEM complete medium. 1 day after transfection, cells are investigated.

1.3.6. Generation of Stable Cell Lines

293 cells are seeded at a density of 500,000 cells/6-well. The next day $CaCl_2$ transfection with linearized and EtOH/acetate precipitated plasmid DNA is carried out as described above. 1 day after transfection cells are trypsinized and reseeded in a 10 cm cell culture Petri dish (about 60 cm^2) and DMEM complete medium is exchanged with DMEM complete medium containing 900 µg/mL G418. About 2 weeks later, colonies that survived the G418 selection are checked by fluorescence microscopy for the expression of the transfected GFP-fusion protein and positive colonies are picked under the microscope (4x magnification objective) under sterile laminar flow by scraping them off with the tip of a pipette, careful suction of the colony and placement into a well of a 24-well plate containing the selection medium. 1 d after the isolation, the adherent colony is separated into single cells by trypsinization and reseeding of the cell suspension in the same well. After reaching confluence, the cells are further expanded up to 75 cm^2 flasks and aliquots are frozen in liquid nitrogen.



1.4. Protein Methods

1.4.1. Cytosolic Cell Extract Preparation

Solutions:

- Nonidet P-40 (NP 40)
- Protease inhibitor cocktail (1 tablet Complete™ protease inhibitor, Roche) in 1 mL PBS makes 50x stock solution): pepstatin, aprotinin, leupeptin, phosphoramidon
- Lysis buffer: PBS def. containing 0.5 % (v/v) NP 40 and protease inhibitors (when the protein concentration had to be determined by Coomassie staining the NP-40 concentration was decreased to 0.2% to reduce interference with the protein quantification).
- Proteasome activity lysis buffer: Tris/HCl 10 mM pH 7.4, 10 % (v/v) glycerol, KCl 150 mM, $MgCl_2$ 10 mM, ATP 5 mM (freshly added prior to use), 0.2 % NP 40
- Adenosintriphosphate (ATP) 100 mM pH 7.4 in PBS complete (PBS deficient containing additionally $CaCl_2$ 1mM, $MgCl_2$ 0.5 mM

Typically cells are lysed with 0.2 % NP 40, protease inhibitors in PBS. Incubation of some minutes is enough to achieve effective lysis. Destruction of the cytoplasm membrane is checked with the inverse microscope (10 x magnification), intact nuclei are visible whereas

the cytoplasmic membranes disappear. After lysis, cell extracts are centrifuged for 15 minutes at full speed, 17 000 x g at 4°C (centrifuge: Heraeus Sepatech Megafuge 1.0 R) to get rid of cytoplasm membrane parts and nuclei.

1.4.2. Protein Quantification (Bradford)

Solutions:

- BSA 1 µg/µL in PBS
- BioRad 1:5 Coomassie Reagent diluted in AD

96-well format: Generation of BSA standard curve: 0 – 5 µg of BSA respectively are pipetted in a well respectively. Cell extract samples are diluted e.g. 1:10 and an appropriate amount is used for quantification in order to stay in the linear range of the assay. 150 µL of diluted BioRad Coomassie Reagent (5x) are added to each well and incubated for some minutes. Absorbance at 595 nm is then measured with an Elisa reader (SLT Lab instruments 340 ATTC). The BSA standard curve is evaluated in MS Excel. According to the equation after linear regression analysis (for the linear range, usually between 0 and 4 µg protein), protein amounts are calculated.

1.4.3. Immunoprecipitation

Solutions:

- 1 % BSA in PBS
- PBS def., 4°C
- Agarose beads: αflag agarose beads (M2-αflag affinity matrix, Sigma, Cat #A-1012), αIKK1/2 agarose beads (Santa Cruz)

Preparation: An appropriate amount of agarose beads with covalently bound antibody (about 15 µl of a 50 % slurry are necessary for one immunoprecipitation) are washed twice with 1 mL PBS (4°C) and centrifuged for 1 minute at full speed in a table top centrifuge (centrifuge: Heraeus Sepatech Megafuge 1.0 R). Blocking: the agarose beads are suspended in 1 mL 1 % BSA in PBS solution and incubated while rotating for 1 hour at 4°C, in order to block unspecific protein binding sites and therefore to increase the specificity. The beads are centrifuged as above and the supernatant is removed. Immunoprecipitation: cell extract and PBS (4°C) are added to the blocked beads and PBS (4°C) is added to a final volume of 800 µL and incubated while rotating for 1 hour at 4°C. Washing procedure: 4x washing with PBS (4°C) and centrifugation are performed as described above. After the third time washing the resuspended beads are transferred to a new reaction tube in order to prevent that proteins that bound unspecifically to the tube are eluted by SDS-buffer. 20 µL of 1x SDS sample buffer are pipetted to the beads and proteins are detached from the beads by heating to 95°C for 5 minutes. After centrifugation for 3 minutes at full speed in a table top centrifuge, the supernatant is used for SDS PAGE (or stored at -80°C for later electrophoresis and Western blotting).

1.4.4. Denaturing SDS-PAGE

Solutions:

- Solution A: 1.25 M Tris/HCl pH 8.8, 0.4 % SDS
- Solution B: 30 % Acrylamide/N', N'-Bismethyleneacrylamide (29:1) in H_2O
- Solution A Stack (ASt): 0.5 M Tris/HCl pH 6.8, 0.4 % SDS, phenol red
- Ammoniumpersulfate (APS) 10 % (w/v) in AD
- N, N, N', N'- Tetra-ethylethylene-diamine (TEMED)
- Butanol
- 1x SDS running buffer: Tris base 25 mM, glycine 250 mM, 0.1 %SDS
- 6x protein loading dye: Tris/HCl 750 mM pH 6.8, 60 mM DTT, 12 % SDS, 60 % glycerol anhydrous, bromophenol blue
- prestained protein marker: Benchmark™ (INVITROGEN)

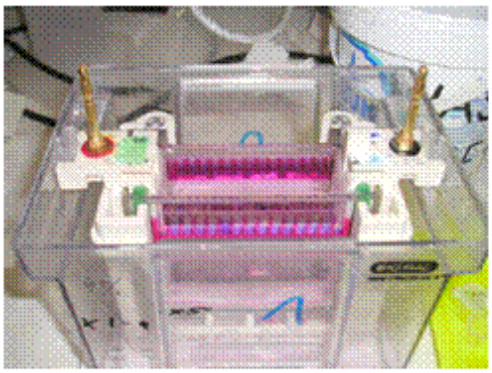


Figure 3. 1: BioRad PAGE electrophoresis apparatus

separating gel SDS PAGE

solution	7.5%	10%	12.5%
H_2O [mL]	4.85	4	3.2
sol. A [mL]	2.5	25	2.5
sol. B [mL]	2.5	3.4	4.2
APS 10% [µL]	50	50	50
TEMED [µL]	10	10	10

For 2 SDS-PAGE gels (6 cm x 9 cm x 0.015 cm) 10 mL of separating gel are prepared. The following table contains the volumes of solutions according to 7.5 %, 10 % and 12.5 % SDS-PAGE gels. H_2O, solution A and solution B are mixed (preventing air bubbles), then APS and TEMED are added in order to start polymerization. The separating gel solution is mixed briefly and is filled in the prepared gel cast system (BioRad Mini Gel system), about 5 cm high (to about 0.5 – 1 cm below the position of the bottom of the sample comb). A layer of butanol is pipetted on top to exclude oxygen from the surface since the presence of oxygen disables polymerization. After about 30 minutes polymerization is finished, the butanol layer is poured off and the stacking gel is layered on top of the separation gel, immediately (to prevent drying of the gel).

For 2 stacking gels 10 mL are prepared. According to the next table H_2O, solution Ast and solution B are mixed, and then APS and TEMED are added in order to start polymerization. The solution is pipetted on the separating gel to the upper edge of the glass plates (until overload, bubbles are swept away like this) and then the combs are placed between the

glass plates avoiding air bubbles. Again, polymerization is finished after 30 minutes, the SDS PAGE gel is then ready for use.

stack SDS PAGE	
Solution	**4 %**
H_2O [mL]	6.1
sol. Ast [mL]	2.5
sol. B [mL]	1.3
APS 10 % [µL]	50
TEMED [µL]	15

Protein samples are mixed with 6x protein loading dye to a final concentration of 1x protein loading dye. Samples are boiled for 5 minutes at 95°C, and then briefly centrifuged for 2 minutes at full speed in an eppendorf table top centrifuge. After polymerization, the gel sandwich is taken out of the gel cast and placed in the electrophoresis tank, adding 1x running SDS running buffer in the inner and outer chamber, the combs are removed and the slots are cleansed from half-polymerized gel pieces by pipetting up and down some running buffer. Then protein samples and prestained protein marker are loaded on the gel. Electrophoresis is started immediately to prevent diffusion of the samples into the surrounding gel area. Electrophoresis parameters: 30 mA per gel for about 1 hour, depending on how big the resolved proteins were.

1.4.5. Electro Transfer Blotting on PVDF Membrane

Solutions

- 1x SDS western transfer buffer (1L): 25 mM glycine (2.9 g), 50 mM Tris base (5.8 g), 20 % methanol (200 mL), 0.4 % SDS, aqua dest. ad 1L, pH should be 8.0
- Methanol 100 %

Activation of PVDF membrane (Millipore, Immobilon Cat. No. #IPVH20200): in order to make the PVDF membrane hydrophilic it has to be soaked in methanol 100 % for 15 seconds, then rinsed in aqua dest. for 2 minutes and equilibrated in Western transfer buffer for at least 3 minutes. The blotting sandwich is built as follows: one blotting sponge lying on blotting cassette soaked in western transfer buffer, two 3MM whatman filterpapers soaked in western transfer buffer, the activated PVDF membrane, the polyacrylamide gel, two 3MM whatman filterpapers soaked in western transfer buffer, one blotting sponge soaked in western transfer buffer. After each layer possible bubbles are removed by rolling a plastic pipet on top of the current layer. The blotting cassette (BioRad) is then closed and placed in the transfer chamber (orientation overview: cathode – / gel / membrane / anode +). Blotting parameters: 150 mA per blotting cassette/gel for 2 hours.

1.4.6. Western Blot

Solutions:

- Ponceau S solution: 0.1 % (w/v) Ponceau S in 5 % (v/v) acetic acid
- Blocking solution: 5 % skimmed milk powder in PBS def.,0.1 % Tween20
- Wash solution: PBS def. 0.1 % Tween20
- Antibody solution: primary or secondary antibody diluted in 1 % skimmed milk powder in PBS def., 0.1 % Tween20
- Primary antibodies: αIKK2 (1:500, mouse IMGENEX), αJAB1/CSN5 (1:10 000, rabbit, Santa Cruz), αflag tag M2 (1:2000, mouse, Sigma), αNEMO/IKKγ (1:500, rabbit, Santa Cruz), α HA tag (1:1000), αIKK1 (1:500, Santa Cruz)
- Secondary antibodies coupled with horseradish peroxidase (HRP) αmouse (1:5000, Amersham-Pharmacia), α rabbit (1:5000, Amersham-Pharmacia)
- NaN_3 solution 20 % (w/v) 200x in AD
- ECL: (Amersham), ECL plus (Amersham)
- Pierce Super signal West pico (Cat. No. #34080)
- Pierce Super signal West femto (Cat. No. #34095)

The PVDF membrane is taken out of the transfer sandwich, possible gel leftovers sticking on the membrane are removed and the membrane is incubated in Ponceau S solution while gently shaking for 5 - 10 minutes. After this, the Ponceau S solution is removed and collected for reuse, the membrane is destained by rinsing with AD until the bands can be clearly seen. At this point, desired lanes can be separated by cutting and trimming the membrane with a sharp scalpel. The stained membrane is scanned on a regular computer-scanner to assess equal protein loading (respectively for normalization purposes). Then the membrane is incubated in blocking solution for 30 minutes while gentle shaking to block unspecific binding sites. Appropriate primary antibodies are diluted in 5 – 10 mL antibody solution in a 50 mL Falcon tube. In order to cover the membrane evenly with antibody solution, the membrane is placed on the inner wall of the 50 mL Falcon tube after the blocking step with the protein side facing the inner side and the antibody solution and incubated on a rotor for 1 hour at 4°C. For reuse the antibody solution is supplemented with NaN_3 20 % (1:200) to a final concentration of 0.1 % NaN_3 to prevent bacterial growth and stored at 4°C. The membrane is rinsed twice shortly with about 20 mL wash solution, then washed twice with about 100 mL wash solution for 5 minutes and washed once with about 100 mL for 15 minutes. After the last washing step, the antibody solution containing the secondary antibody is incubated under the same conditions as above for 1 hour. The washing protocol is the same (2 x rinsing, 2 x 5 minutes, 1 x 15 minutes). Detection of immunoblotted proteins is done by chemiluminescence reaction with substrate solutions provided by Pierce or Amersham-Pharmacia. The membrane is incubated with the substrate solutions according to the manufacturer's protocol. (ECL: (amersham) mixing ratio of solution 1 and 2 1:1 incubation time 1 minute, ECL plus mixing ratio of solution A and B 1:40, incubation 5 minutes, Pierce Super signal West pico: mixing ratio of solution

1 and 2 1:1 incubation time 5 minutes, Pierce Super signal West femto mixing ratio of solution 1 and 2 1:1 incubation time 5 minutes). The membrane is taken out of the solution and is placed in a plastic wrap avoiding wrinkles of the wrap. Then excess of substrate solution was removed by streaking from the outside with a paper towel. Depending on signal intensity the blot is exposed to Kodak X-OMAT or BioMax films or a CCD-camera based detection system (LumiImager, Roche) is used for detection of chemiluminescence. The distances of the protein markers from the top of the separation gel are measured and used for generating a standard curve of the molecular weights (using the correlation between the log of the MW and the migration distance; calculations and regression analysis are done with MS Excel).

1.4.7. Silver Staining of PAGE Gels

Solutions

- Fixing solution: 50 % ethanol, 10 % glacial acetic acid, ad 100 % with aqua dest.
- Incubating solution (1L): 30 % ethanol, sodiumthiosulfate anhydrous 2g, sodiumacetat anhydrous 34 g, fill up to 1L with aqua dest. Before use add 125 µL of glutaraldehyde/50 mL incubating solution.
- Silvernitrate solution (1L): $AgNO_3$ 1 g, dissolved in 1L aqua dest.. Before use add 10 µL of formaldehyde/50 mL of silver nitrate solution.
- Developing solution (1L): Na_2CO_3 anhydrous 25 g, dissolved in 1L aqua dest.. Before use add 10 µL of formaldehyde/50 mL of developing solution.
- Stop solution (1L): sodium-EDTA 15.78 g dissolved in 1L aqua dest..

After electrophoresis, the polyacrylamide gel is taken out of the casting sandwich and placed in a clean glass beaker filled with fixing solution. All following steps are carried out while gently shaking. The gel has to be incubated with the fixing solution for 30 minutes. After fixation an appropriate amount of incubating solution including glutaraldehyde (the gel has to be at least covered by liquid) is prepared and added to the gel, followed by incubation for 15 minutes, discarding the fixing solution and washing with aqua dest. 3x for 5 minutes and 10 minutes incubation in silvernitrate solution including formaldehyde. The silvernitrate solution is collected (special waste). Developing is carried out by incubating the gel in developing solution including formaldehyde until the desired intensity of protein staining is reached, followed by discarding of developing solution and adding stop solution. The gel should incubate for at least 1 hour in the stop solution. Afterwards the gel can be stored in aqua dest. or dried with vacuum.

1.4.8. Stripping of PVDF Membranes

Solutions

- Stripping buffer: 62.5 mM Tris/HCl pH 7.0, 2 % (w/w) SDS, 0.7 % mercaptoethanol

For reprobing PVDF membranes the previous antibody has to be removed by a socalled stripping procedure. An appropriate amount of stripping buffer (about 25 ml per blot) is

prewarmed to 50°C, the membrane is laid on the inner wall of a hybridization tube and together with the stripping buffer it is incubated under rotation at 50°C in a hybridization oven for 30 minutes. After stripping, the membrane is washed twice for 10 min in PBS/0.5% Tween 20 and blocked again (by incubation in 5 % milk powder in PBS-Tween). It is then ready for the next immunoblotting.

1.4.9. Immunoprecipitation of Proteasomes

Solutions:

- Proteasome activity lysis buffer: Tris/HCl 10 mM pH 7.4, 10 % (v/v) glycerol, KCl 150 mM, $MgCl_2$ 10 mM, ATP 5 mM pH 7.4 (freshly added prior to use), 0.2 % NP 40
- Proteasome wash buffer: Tris/HCl 10 mM pH 7.4, 10 % (v/v) glycerol, KCl 150 mM, $MgCl_2$ 10 mM, ATP 5 mM (freshly added prior to use)
- Proteasome elution buffer: Tris/HCl 10 mM pH 7.4, 10 % (v/v) glycerol, KCl 2 M, $MgCl_2$ 10 mM, ATP 5 mM pH 7.4 (freshly added prior to use)
- α20S beads: antibody against subunit α4 coupled to agarose beads (Affinity Ltd, UK, PW 9005, Batch Z04562)
- NaN_3 10 mM in PBS def.

A blue p1000 tip was plugged with a p200 filter, then 0.5 mL of αsubunit α4 coupled to agarose beads were placed on the filter. The generated small column is equilibrated with 3 x 1 mL proteasome wash buffer by gravity flow. 293 or HeLa cells are lysed with proteasome activity lysis buffer and precleared as described above under “cell extract generation”. The cell extract is loaded on the column by gravity flow, the column is washed with 3 x 1 mL physiological proteasome wash buffer. Elution is performed with 3 x 1 mL high salt proteasome elution buffer. Elution fractions are collected and investigated for proteasome activity with the fluorogenic substrate Suc-LLVY-AMC. The proteasome fractions are either dialysed or TCA precipitated. The column is reequilibrated with proteasome wash buffer and stored in PBS def. containing NaN_3 to prevent bacterial growth.

1.4.10. Dialysis

Solutions:

- Dialysis buffer: Tris/HCl 10 mM pH 7.4, 10 % (v/v) glycerol, NaCl 100 mM, $MgCl_2$ 10 mM, ATP 5 mM pH 7.4 (freshly added prior to use)

Samples with salt concentrations of 2 M are transferred to a reaction tube, the lid was removed and instead a dialysis membrane (cut off) is fixed. This small dialysis chamber was fixed up side down on the wall of a big glass beaker filled with about 300 mL dialysis buffer. Dialysis was carried out o/n at 4°C.

1.4.11. Glycerol Gradient Centrifugation

Preparing: a glycerol gradient of 35 – 80% glycerol is used. Glycerol dilutions from 35 - 80% are prepared in 5 % steps. Thick walled centrifugation tubes are placed upright in a rack over night at –80°C. For pouring the gradient, 120 µL of each glycerol dilution are pipetted in the centrifugation tube starting with the heaviest (80% glycerol). After each dilution the rack is placed back at –80°C for 2 minutes until the layer is either very viscous or frozen. Then the next layer is pipetted on top and so forth. With that procedure a very sharp gradient can be created. On top of the last frozen layer the sample of 120 µL are pipetted. Then all tubes are balanced on an analysis balance.
Centrifugation parameters (Beckmann Ultracentrifuge Optima TLX): 40000 rpm, 4°C, vacuum, 16 hours. After centrifugation, the gradient was fractionated manually with a Gilson p200 120 µL steps each.

1.4.12. Native PAGE

Solutions:

- Native separating gel buffer / running buffer: 0.18 M Tris/borate pH 8.3, $MgCl_2$ 5 mM, DTT 1 mM, 1 mM ATP
- Acrylamide/N', N'-bismethyleneacrylamide mix: 37.5:1
- Ammoniumpersulfate (APS) 10% w/w
- N, N, N', N'- Tetra-ethylmethylen-diamine (TEMED)
- Adenosintriphosphate (ATP) 100 mM pH 7.4 in PBS complete
- Native loading dye: 50 % glycerol containing 0.0025 % xylene cyanol

Protocol (Glickman et al. 1998a) slightly modified

The native PAGE gels containing 4 % acrylamide/bisacrylamide are poured without a stack, for 2 gels (6 cm x 9 cm x 0.015 cm) 25 mL of separating gel are prepared as follows: 21.3 mL native separating gel buffer and 3.7 mL acrylamide/bisacrylamide mix are mixed thoroughly avoiding bubbles, 250 µL 10 % APS and 25 µL TEMED are added to start polymerization. The solution is poured immediately into the prepared gel cast cassette and the combs are inserted. After 30 minutes when polymerization is finished, the gel sandwich is placed in the electrophoresis tank and running buffer is poured in both electrode chambers. 20 µL samples are mixed with 5 µL of loading dye and loaded on the gel. Electrophoresis parameters: 30 mA for 1 gel. The gel is running for about 2 hours until the blue front eluated into the lower running buffer chamber. In most cases, the native gel is afterwards subjected to a proteasome activity overlay assay.

1.4.13. Proteasome Activity Assay

Solutions

- Fluorogenic peptide solution: Suc-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-AMC) 1 mg/mL in N,N –Dimethylformamide (100x)
- Proteasome activity buffer: Tris/HCl 10 mM pH 7.4, KCl 140 mM, $MgCl_2$ 5 mM, ATP 5 mM

10 µL of cell extract are mixed with 90 µL proteasome activity buffer and 1 µL of Suc-LLVY-AMC solution and vortexed well. With a 1 mL syringe the sample is injected bubblefree into the fluorescence detector (model: FP-920 Jasco Inc, Japan). The measuring parameters of the acquisition method are: Time scan for 5 min at an excitation wavelength of 380 nm and emission wavelength of 440 nm with data acquisition every 5 seconds. The gain is set to 10 (2^{nd} stage of 4 logarithmic amplification steps) and the emission bandwidth is set to 18 nm slit. Due to proteasome activity, the fluorescent molecule AMC (7-amino-4-methyl coumarin) is released from the non-fluorescent peptide substrate Suc-LLVY-AMC resulting in a linear increase of fluorescence over time with the slope being proportional to proteasome activity. The resulting graph of proteasome activity is then exported to MS Excel and evaluated. The slope of the fluorescence increase is calculated by linear regression and normalized by total protein amount to determine the normalized proteasome activity. It has to be noted that the fluorogenic peptide substrate is cleaved by proteasomes independent from ubiquitination and most likely also independent from the proteasome activator complex.

1.4.14.Proteasome Activity Overlay Assay

Solutions:

- Fluorogenic peptide solution: Suc-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-AMC) 1 mg/mL in N,N –Dimethylfomamide (100x)
- Proteasome activity buffer: Tris/HCl 10 mM pH 7.4, KCl 140 mM, $MgCl_2$ 5 mM, ATP 5 mM

After electrophoresis, the native 4 % PAGE gel is carefully taken out of the gel sandwich and placed on a plastic wrap. About 5 mL of overlay solution containing 5 mL proteasome activity buffer and 50 µL of Suc-LLVY-AMC solution arere spread evenly over the surface of the native gel and the gel is soaked for about 10 minutes. Fluorogenic peptide activity is detected with the Sybr green filter of the LumiImager™ (Roche) detection system.

1.4.15.Trichloro Acetic Acid Precipitation

Solutions:

- TCA 50 % w/w
- Acetone

The samples are mixed with trichloro acetic acid solution (TCA) to a final concentration of 10 % w/w. After incubation for 1 hour at 4°C the samples are centrifuged for 30 minutes at

17.000 x g at 4°C in a tabletop centrifuge. The supernatant is removed and the pellet is washed once with cold acetone – followed by drying for 5 min and uptake in the appropriate buffer (e.g. SDS-PAGE buffer).

1.4.16.Kinase Assays with COP9 Complex as Substrate

Solutions

- Kinase lysis buffer: Hepes 10 mM pH 7.9, β-Glycerophosphate 20 mM, Na-Orthovanadate 1 mM, NaF 1 mM, DTT (dithiothreitol) 1 mM, Na-EDTA 2 mM, NaCl 150 mM, 1 % Triton X 100, rest of volume aqua dest.
- Kinase buffer: Hepes 10 mM pH 7.9, β-glycerophosphate 12.5 mM, $MgCl_2$ 5 mM, NaF 1 mM, DTT 1 mM, ATP 50 µM pH 7.4, NaCl 50 mM, rest of volume aqua dest. Add freshly before use: $MnCl_2$ 1 mM
- ^{32}P γ-ATP 10 µCi/µL (Amersham)

Immunoprecipitation of flag IKK2wt with M2 agarose beads is carried out as described above. The beads are washed 2x with cold PBS and 2x with kinase buffer and the kinase buffer is quantitatively removed by a capillary Pasteur pipette or a thin electrophoresis application pipette tip. Per sample 10 µL kinase buffer and 25 µCi ^{32}P γ-ATP (2.5 µL) are mixed and preincubated for 10 minutes at 37°C. 12.5 µL of this preincubated kinase buffer mix and 8 µL COP9 substrate or 1 µL IκBα substrate are pipetted on the wall of the reaction tubes containing the immunoprecipitation sample. The solutions are briefly centrifuged and mixed quickly in order to avoid contact between kinase and substrate without ^{32}P γ-ATP. The kinase reaction is carried out for 2 hours at 37°C and is stopped by adding 6x protein sample buffer. Then the kinase samples are subjected to 12.5 % SDS PAGE, silver staining is performed, the silver-stained gel is vacuum-dried and exposed to phosphor screens or Kodak X-OMAT films.

1.4.17.Detection of ^{32}P γ-ATP Phosphorylated Proteins

Fixed and silver-stained SDS-PAGE gels are sandwiched between a layer of Whatman filter paper and plastic wrap, the sandwich is placed in the vacuum dryer with the wrap layer facing up. The gel is dried for about 15 minutes at 80°C with vacuum, and then the dried gel is cooled down between two heavy objects to decrease deformation and cracking. The dried gel covered with a plastic wrap is exposed to a phosphor screen o/n or to Kodak films for some days depending on signal intensity. The phosphor screens are scanned with Storm™ equipment (Molecular Dynamics).

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1.5. GFP, YFP and CFP: the Living Color Family

The green fluorescent protein (GFP) was discovered in the jellyfish Aequorea victoria (figure 3.2) as a protein fluorescing without the need for any substrates or coenzymes. The

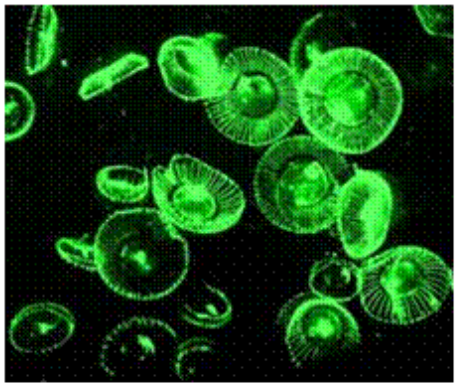

Figure 3. 1: the jellyfish Aequorea victoria

organism A. victoria contains both GFP and the chemiluminescent protein aequorin. Upon light emission by aequorin GFP absorbs this light and fluoresces green. As soon as the GFP gene was cloned and successfully expressed in non-jellyfish organisms the way was clear for GFP to use it as tracking tool fused to various proteins of interests.

Fluorescence is a phenomenon of molecules that absorb light of a certain wavelength and emit light of a longer wavelength. Absorption of light is also called excitation, whereas this energy is not kept within the molecule but is emitted again. Emission wavelengths are always redshifted by that means nearer to the red spectrum and a longer wavelength than the excitation wavelength. Figure 3.3 shows an example of excitation and emission spectra.

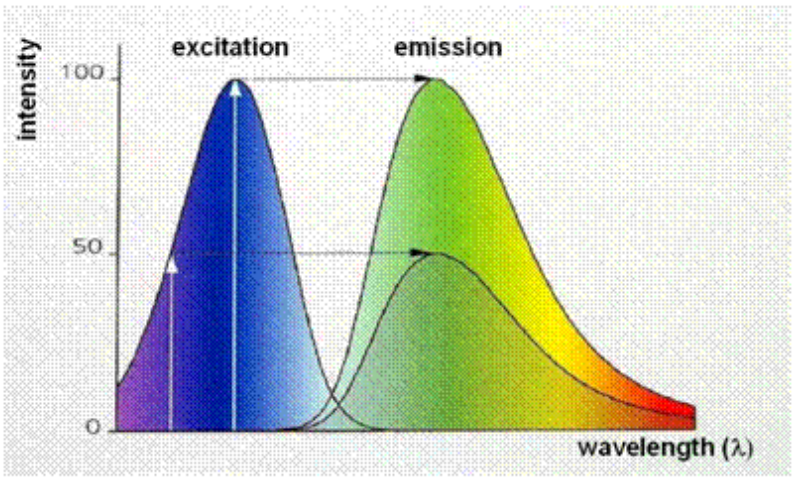

Figure 3. 1: scheme of fluorescence: excitation and emission spectra

GFP has a molecular weight of 28 kDa. Crystal structure data of GFP dimers reveals a barrel like structure composed of β-sheets designated as β-can structure. (figure 3.4) However, some biochemical properties of wt GFP such as folding, dimerization, absorbance peaks, fluorophore formation and expression levels were not satisfying. Therefore amino acid mutations were generated in order to improve the properties of wildtype GFP (wtGFP). The evolved GFP with “special features” was then named enhanced GFP (EGFP). EGFP has a much faster fluorophore formation, 0.45 hours instead of 2 hours and it produces a much brighter and stable GFP (mutation Ser^{65} →Thr^{65} about 35-fold brighter) and it could be expressed correctly folded at 37°C. Protein dimerization at high expression levels could be diminished with mutations like Ala^{206} → Lys^{206}, Leu^{221}→ Lys^{221} or Phe^{223} → Arg^{223}. Wild type GFP possesses two absorbance peaks which are reduced to one in EGFP to increase specificity of excitation (mutation Ser^{65} →Thr^{65}, Ala^{65}, Gly^{65}, Cys^{65} or Leu^{65}). WtGFP’s temperature sensitivity could be removed by introduction of the mutation Phe^{64} → Leu^{64}.

Figure 3. 1: β-can crystal structure of a GFP dimer

The use of EGFP as tracking molecule offers the possibility to study living systems with a non-destroying approach, given the fact that EGFP does not interfere with the properties of the protein of interest. Certainly it has to be tested whether the EGFP-fusion protein behaves like the untagged one, but in most cases EGFP is only little interfering if at all.

EGFP fusion proteins are generated by simply cloning the GFP gene up- or downstream of the gene of interest in an appropriate DNA construct. To investigate how the GFP-fusion protein is behaving in living cells, transfections are carried out and 24 hours after transfection cells can be investigated by fluorescence microscopy, flow analysis or other methods based on fluorescence. Comparisons of GFP-chimeric proteins with wild type, untagged proteins can be done with reporter gene assays, immunofluorescence as compared to GFP-fluorescence or other appropriate biological tests. In addition to the development of enhanced, improved GFP variants, other fluorescent protein versions were generated that differ from GFP in the color of the emitted light (and also the excitation wavelength)

Blue (Tyr^{66} → His^{66}), cyan and yellow fluorescent mutants with different absorbance and emission profiles were obtained (see figure 3.5). This opened the possibility to track two (or in special applications even more) fusion proteins with different GFP variants simultaneously in one cell.

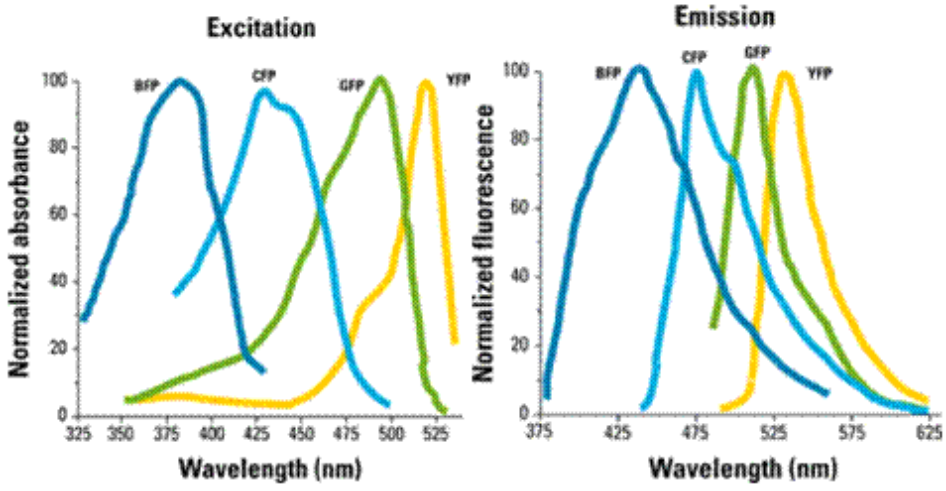

Figure 3. 1: Excitation and emission spectra of enhanced blue, cyan, green and yellow fluorescent protein

1.6. Confocal laser scanning microscopy

Confocal microscopy differs from conventional light microscopy in the light source, the detection, generation and resolution of acquired images. In a conventional fluorescence microscope the whole specimen is illuminated by a certain excitation wavelength, likewise

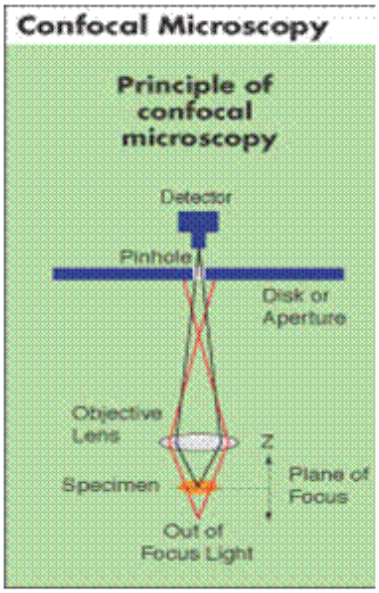

Figure 3. 1: raypath in confocal microscopy

emitted light is gathered from all planes of the specimen. Visible light cannot be focused in a single plane, therefore not only emitted light from the focal plane but below and above the specimen are detected. This additional light detected results in a blurry image of decreased contrast and resolution, especially for thick specimens. In contrast to conventional light microscopy, in confocal microscopy a defined spot in the focal plane of the specimen is illuminated at a certain time point. Laser light sources of defined excitation wavelengths are used. The laser light is focused by passing through a very small aperture, such as a pinhole or a slit. Furthermore, emitted light from below or above the focal plane is eliminated by preventing passing a second pinhole (see figure 3.6, orange lines). By changing the z-axis distance between specimen and objective lense, the focal plane can be adjusted and so called optical slices of the specimen in X-Y plane can be generated. This technique is a non-invasive approach to investigate both fixed and living cells. Emitted light of an illuminated spot in the focal plane is detected by photomultiplier tubes (PMT), which enhance signal. Series of illuminated PMT outputs are processed to an image.

1.6.1. Fluorescence Microscopy of Fixed Cells

Solutions:

- 4 % (w/v) paraformaldehyde pH 7.4 in PBS
- mounting medium: glycerol in PBS (1:6)
- round glass cover slips (∅ 15 mm) , sterile, stored in 70 % ethanol

293 or HeLa cells are passaged and seeded at a density of 500.000 cells/6-well (40 % confluence), each 6-well contains a round sterile glass cover slip. 1 day after lipofectamine or $CaCl_2$ transfection of 293 or HeLa cells with fluorescent fusion proteins, the culture medium is removed, the cells are washed once with PBS def. and about 2 mL 4 % paraformaldehyde solution per 6-well are carefully pipetted to the cells. After about 10 minutes incubation at room temperature the paraformaldehyde solution is removed. The fixed cells are washed with PBS and covered with mounting medium. Then the coverslip is carefully taken out of the 6-well and placed on a glass slide with the cells facing the slide, trying to avoid bubbles. Excess of mounting medium is removed and clear nailpolish is applied on the edges of the coverslip to seal the fixed sample to prevent drying. Fluorescent microscopy is then performed with the sample.

1.6.2. Live Cell Fluorescence Microscopy

- greased aluminum slide

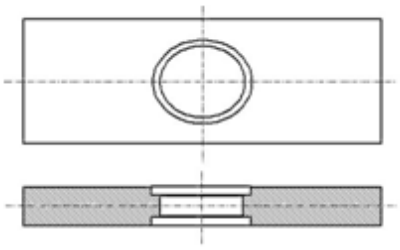

Figure 3. 1: aluminum slide used for live microscopy

293 or HeLa cells are seeded in 6-well plates containing round sterile coverslips (diameter: 15 mm), the next day lipofectamine or $CaCl_2$ transfection is carried out. 1 day after transfection the round coverslips are carefully taken out of the well and pressed on the hole of an aluminum slide (figure 1.7). The edges of the aluminum slides are greased to seal the cover slips. About 70 µL of DMEM complete medium is pipetted on the cells on the coverslip. With a second blank coverslip pressed on the other side of the aluminum slide a chamber filled with medium is created. The aluminum slide is placed on the object table with the cells grown on the coverslip on the bottom.

1.6.3. Fluorescence Recovery after Photobleaching (FRAP) studies

Figure 3. 1: fluorescence laser scanning microscope Zeiss LSM 510

The FRAP technique allows to investigate molecular dynamics of fluorescent proteins like EGFP in a living cell being a non invasive microscopy technique. This microscopy technique consists of 4 steps: a pre-bleach scanning of the whole cell, bleaching of a pre-defined region with 100 % laser intensity, a time series scanning and a post bleach scanning image. The pre and post bleach images are later used for calculation of corrected FRAP. During a bleaching process laser light of high intensity irreversibly destroys the fluorophore and significantly decreases the mean fluorescence in the defined bleach region. Then a set of scan images are taken to observe the increase of fluorescence (recovery) due to diffusion processes in and out of the scan region (FRAP scheme see figure 3.9). The diffusion velocity of the fluorescent protein is indirect proportional to its molecular weight. The recovery behavior of a fluorescent protein after a bleaching process is typical for its cellular property. Proteins can diffuse rather undisturbed or are incorportated in larger complexes and structures.

FRAP gives information about relative amounts of mobile and immobile fractions of the fluorescent protein abundant in the cell. First an image of the whole cell is captured before bleaching. Both, a round scan and bleaching area in the cell are defined. The following

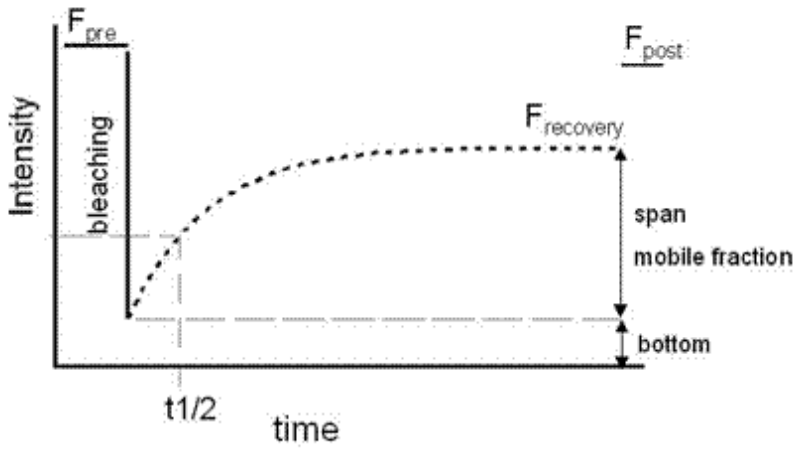

Figure 3. 1: scheme of a FRAP experiment, fluorescence intensities are monitored over time

acquisition of time series are performed: 1 image before bleaching, 70 iterations of bleaching with 100 % laser power, 100 scans of the bleach area with averaging of 4 scans are taken. An image of the whole cell is captured after the bleaching and recovery process. The image series are exported to TIF files with LSM Image Examiner and later evaluated with Scion Image. The bleach region in each image of the time series is measured for its mean fluorescence using the “measure all” command of the Measure Macro of Scion Image. Resulting mean fluorescence data is transferred and evaluated in MS Excel and Graphpad Prism. Fluorescence recovery data have to be corrected due to co-bleaching of the whole cell, by that means the total cell mean fluorescence decreases and therefore a recovery of e.g. 100 % would never be achieved even though the fluorophores are absolutely mobile. The FRAP correction factor is obtained by the ratio of total cell fluorescence $F_{post}/F_{pre} = F_{corr}$. All fluorescence recovery intensities (except for prebleach scan) have to be divided by this correction factor. The recovery period could be modelled best with a one phase exponential equation:

Y [%fluorescence)= Span * (1- e^{1-kx}) + Bottom

This curve starts at the bottom and increases to bottom + span (= plateau) with a rate constant K. The half life is calculated with $t_{1/2}$= ln2/K or $t_{1/2}$= 0.693/K.