Chapter 2

MATERIAL AND METHODS

2.1 Cell culture

2.1.1 Equipment and cultivation

Cell cultures were handled on a clean bench (HERAsafe® from Heraeus®). Cells were cultured in Tissue Culture Petri Dishes (TPP, Switzerland) in HERAcell® CO^2-incubators (Heraeus®) at a constant temperature of 37°C and a minimum relative humidity of 80%. Cell culture media was D-MEM containing 1000 mg/L D-Glucose, sodium pyruvate, 25 mM HEPES and phenol red (GIBCO). Fetal calf serum (FCS, GIBCO), heated to 56°C for 45 min and sterile-filtered through 0.2 µm membrane (Stericup™ and Steritop™ for 500 ml, Millipore) was added to the media (final 10 % per volume). Penicillin/streptomycin (Invitrogen™) and extra L-glutamine (GIBCO) was added to final concentration of 1% per volume.

Cells were seeded out every 3 to 4 days at a density depending on the cell type (for HEK 293T about 2 x 10^5 cells per 145 cm^2 cell culture plate). Cells were washed with PBS and detached from culture dishes using a mixture of proteases (Accutase™, PAA). To inhibit Accutase™ the same amount of media was added to the detached cells. Cells were then centrifuged for 5 min at 2000 rpm. Supernatant was removed by vacuum and the cell pellet was resuspended in fresh media. To count the cells using a haemocytometer (Neubauer counting slide) 5-15 µl of the cell solutions were first diluted with trypan blue 0.4% (SIGMA) to 1:2 or 1:4. With this reagent, living cells (white) are easy to distinguish from dead cells (blue).

For storing over a long time cells were frozen in 2 ml cryo tubes (GREINER-bio-one) in freezing-solution composed of 90% FCS and 10% dimethyl sulfoxide (DMSO; SIGMA). Before putting in liquid nitrogen (–196°C) tubes were pre-cooled at –80°C (allowing 1°C/min for cooling). For re-thawing the tubes were warmed up in a 37°C water bath, and pre-warmed media was added slowly before centrifuging and resuspending in fresh media. Contamination with mycoplasma was regularly checked using "PCR Mycoplasma Detection Set" (TAKARA BIO INC.).

One to two days before reverse transfection, cells were seeded out in a 60 cm^2 cell culture plate (145 cm^2 plate for HeLa cells) at cell density depending on cell type and added on top of arrayed slides at transfection day in a defined amount, also depending on the cell type (see section 2.1.2).

2.1.2 Cell lines

HEK 293 (human embryonic kidney) was first established in 1977 as a permanent adherent cell line of human embryonic kidney after transformation with human adenovirus type 5 (Graham et al., 1977).

HEK 293T is an HEK 293 derived cell line that expresses the SV40 large T-antigen. Expression of the T-antigen can enhance proliferation of cultured human cells (Bednarz et al., 2000; Kahn et al., 1993).

HEK 293 was stable transfected with the reporter pGAL/lacZ to create the cell line HEK 293-LZ. Selection media contained Zeocin™ Selective Reagent (Invitrogen™) at a final concentration of 300 ng/µl. Though HEK 293T transfection efficiencies are higher (see above), HEK 293 had to be used to generate stable transfections because of resistance in HEK 293T.

For the same reason, HEK 293 was also stably transfected with pBD-LBD to create the bait-containing cell line HEK 293-LBD and with pBD-SMRT to create HEK 293-BD-SMRT. Transfected cells were selected by geneticine disulphate G-418 (Promega) at a final concentration of 1000 ng/µl. Except for the addition of neomycin in the media, HEK 293 cell lines were treated the same as HEK 293T cells, e.g. number of cells per slide for reverse transfection. During reverse transfections, G418 was omitted to provide optimum conditions for growing and expression.

Various other adherent cell lines were tested for their suitability for reverse transfection experiments: PC-3 (human prostate), WI-38 (human lung fibroblast), HeLa (human cervical carcinoma cells transformed by human papilloma virus 18 (HPV18)), COS 7 (African green monkey kidney cells), HepG2 (human hepatocellular carcinoma), and Hekl (human skin fibroblast).

WI-38, HeLa, COS 7, HEK 293 and its variant HEK 293T were from ATCC. PC-3 was from the German Collection of Micororganisms and Cell Cultures (Braunschweig). HepG2 was a kind gift from Dr. S. Sperling, and Hekl was a kind gift from Prof. Monica Hirsch-Kaufmann (both MPIMG, Berlin). Media in all cases was D-MEM (GIBCO®) as described before.

Cell lines were only used for reverse transfection experiments up to passage P20, carefully preventing overgrowing during culture. One day before transfection 1x 10^7 cells of HEK 293T, HEK 293, HEK 293-LBD, PC-3, WI-38 and HepG2 and 5 x 10^6 cells of COS 7 were seeded out in a 60 cm^2 cell culture plate. For HeLa 5 x 10^6 cells were pre-cultured in a 145 cm^2 plate. When cells were seeded out 2 days before transfection, the number of cells seeded out was halved. On the day of transfection, cells were seeded at 3.5 x 10^6 (HEK 293T, HEK 293, HEK 293-LBD and HepG2), 3 x 10^6 (PC-3, COS 7 and WI-38), and 1 x 10^6 (HeLa and Hekl) cells per slide in 8 ml complete media in Quadriperm boxes (Vivascience) (see section 2.3). For hormone-dependent interactions the media was supplemented with methyltrienolone (R1881, Perkin Elmer), medroxyprogesterone acetate (MPA, Schering-AG) and hydroxyflutamide (OH-Flu, Schering-AG).

2.2 Slides

Various commercial and self-made slides were compared for reverse transfection experiments (see section 3.1.1). Slides were from Corning®, TeleChem`s ArrayIt™, Electron Microscopy Science and Scientific Device Laboratory. Reagents used for self-made slides were poly-L-lysine from SIGMA Diagnostics® (P8920), Silane (3-Aminopropyl-triethoxysilane) from PIERCE (80370), and VECTABOND™ from VECTOR Laboratories (SP-18000) Table 1 gives an overview of tested slides.

Table 1
Tested slides and their sources.

Slide type	Source
GAP™ coated slides	Corning®
Poly-L-Lysine slides PL-25C	TeleChem`s ArrayIt™
Poly-L-Lysine slides 63410	Electron Microscopy Science
Poly-L-Lysine slides 067	Scientific Device Laboratory
Poly-L-Lysine slides	Self-made
Silanated slides CSA-25	TeleChem`s ArrayIt™
Silanated slides 63411	Electron Microscopy Science
Silanated slides 068	Scientific Device Laboratory
Silanated Poly-L-Lysine slides	Self-made
VECTABOND™ coated slides	Self-made
Silanated Poly-L-Lysine slides with VECTABOND™	Self-made
Poly-L-Lysine slides with VECTABOND™ = VPL slides	Self-made

As the result of this comparison VPL slides (covered with poly-L-lysine and VECTABOND™ Reagent (Vector Labs)) offered the best choice regarding the cost per slide and efficiency of transfection. These slides were used for further experiments.

VPL slides were made by treating standard 25 x 75 x 1.0 mm slides or standard 10 x 10 x 1.0 mm coverslips as follows:

1. 2 hrs shake in cleaning solution in a glass container
   (70 ml NaOH 1.75M + 160 ml Bidest + 240 ml ethanol 100%)
2. 3 x 5 min wash in Bidest
3. 5 min in acetone, let slides shortly dry
4. 5 min in VECTABOND™ solution
   (7 ml VECTABOND™ Reagent + 350 ml acetone)
5. 3 x 30 sec dip in Bidest
6. Dry at 37°C
7. Put into plastic container with poly-L-lysine solution
   (20 ml poly-L-lysine + 20 ml PBS + 160 ml Bidest),
8. 45 min shake at 4°C
9. Shortly wash in Bidest
10. Dry at 55°C, store in the dark and under vacuum

2.3 Containers for reverse transfection

Normal cell culture substrates are treated for optimal adhesion of the cells. In case of reverse transfection the cells have to stick on the slide and not on the cell culture dish. Hence non-treated cell culture dishes were used that would hold the slide well. Originally a square dish (Becton Dickinson, Figure 1a) was used to reverse transfect 3 slides simultaneously (Ziauddin and Sabatini, 2001).

Eventually, a more flexible system allowing different numbers of slides to be used and allowing the simultaneous transfection of different cell lines was used. This Quadriperm box from Vivascience (Figure 1b) is a non-treated dish with space for one to four slides per box. Using HEK 293T, 1x 10^7 cells for 3 slides are necessary for one square dish, in the Quadriperm 3.5 x 10^6 cells per folder and slide are required. When using coverslips instead of slides, small petri dishes are suitable containers with around 5 x 10^6 HEK 293T cells per coverslip.

Figure 1
Suitable containers for reverse transfection. The square dish from Becton Dickinson (Figure 1a) is well-suited for 3 slides to transfect at the same time, but the Quadriperm box from Vivascience (Figure 1b) with four separate compartments is more flexible. Both containers are non-treated for cell-culture.

2.4 Plasmids

2.4.1 Mammalian two-hybrid kits

Initially, the TOPO® Tools Mammalian Two-Hybrid Kit from Invitrogen™ was used for optimising CAPPIA. Because positive control from this kit was very low even after optimisation Mammalian Two-Hybrid Assay Kit from Stratagene was tested and found to be preferable. Table 2 lists the components of the kits. Both mammalian two-hybrid kits contain constructs for creating baits (including binding domain) and preys (including activation domain), a reporter plasmid and various control plasmids.

Activation domains used in two-hybrid experiments can be either derived from the galactose expression activating yeast GAL4 protein (Chien et al., 1991) or the herpes simplex virus VP16 protein (Dalton and Treisman, 1992; Dang et al., 1991). The transcriptional activation activity of VP16 in mammalian cells can be localised to amino acids 411 to 490 (Triezenberg et al., 1988). Alternatively the activation domain can be derived from nuclear factor - kappaB (NF-κB) (used in the kit from Stratagene). This eukaryotic transcription factor is a member of the family of structurally and functionally related proteins regulating several cellular alterations. It is localised in the cytoplasm of most cells in an inactive form, complexed to IκB inhibitor (Baeuerle and Baltimore, 1988a, 1988b). A number of agents can stimulate the dissociation of the complex and the subsequent translocation of NF-κB to the nucleus, where NF-κB binds to DNA and activates transcription of a number of genes. NF-κB has different subunits, of which e.g. p65 has been shown to be necessary *in vitro* for IκB to inhibit the DNA-binding activity of NF-κB (Baeuerle and Baltimore, 1988b, 1989; Ghosh and Baltimore, 1990; Nolan et al., 1991).

In most two-hybrid systems, the DNA-binding domain is derived from GAL4 protein (see above) or from the *E. coli* protein LexA (Vojtek et al., 1993; Zervos et al., 1993). As an universal activator with both DNA binding and activation function, GAL4 activates transcription of every gene flanked by GAL4 binding sequences (Fischer et al., 1988; Ma et al., 1988; Ptashne and Gann, 1990). The DNA binding and activation domains of GAL4 are known to be separable (Keegan et al., 1986). This knowledge was first used to develop the yeast two-hybrid system (Fields and Song, 1989).

The most common reporter in both yeast and mammalian two-hybrid assays is the *E. coli* lacZ gene. Others include selectable yeast genes like LEU2 (Zervos et al., 1993) or the luciferase gene. Detailed information is summarised elsewhere (Phizicky and Fields, 1995).

Table 2
List of the components of the mammalian two-hybrid kits from Invitrogen™ and Stratagene. Both kits contain plasmids for constructing baits, preys and reporter as well as positive and negative controls.

Component	TOPO® Tools Mammalian Two-Hybrid Kit Invitrogen™	Mammalian Two-Hybrid Assay Kit (Stratagene)
Binding domain	pSV40-GAL4 5`element + SV40 pA 3`element	pCMV-BD (GAL4 binding element)
Activation domain	pSV40-VP16 5`Element + SV40 pA 3`element	pCMV-AD (NF-κB activation element)
Reporter plasmid	pGAL-LacZ	pFR-Luc
Positive control plasmid (binding + activation function)	pCR®2.1/GAL4-VP16	pBD-NF-κB
Control bait plasmid	pCR®2.1/p53	pBD-p53
Control prey plasmid	pCR®2.1/LgT	pAD-SV40T
Negative control prey plasmid	pCR®2.1/VP16-CP	pAD-TRAF

Positive control plasmids pCR®2.1/GAL4-VP16 (Invitrogen™) and pBD-NF-κB (Stratagene) code for proteins that can activate the reporter plasmids without any partner. Other control plasmids of the kits code for a known protein fused to the binding domain (bait) that can interact with another known protein fused to the activation domain (prey) (see section 1.4.1). For example plasmids pCR®2.1/p53 and pCR®2.1/LgT (Invitrogen™) or plasmids pBD-p53 and pAD-SV40T (Stratagene) are co-transfected and expressed in mammalian cells. The fusion proteins will interact and activate the corresponding reporter construct. Plasmid pBD-p53 expresses GAL4 DNA binding domain and a hybrid protein of murine p53, an anti-oncogen, which plays a role in the onset of cell cycle arrest or apoptosis following DNA damage. Plasmid pAD-SV40T expresses a hybrid protein containing NF-κB transcription activation domain fused to parts of simian virus 40 large T

antigen (SV40T). Since 1979 it has been known that p53 binds to SV40T (Lane and Crawford, 1979). Later Li and Fields used yeast two-hybrid system to identify modified binding ability of different mutations in p53 to SV40T and showed at the same time the high sensitivity of two-hybrid system for the identification of protein interactions (Li and Fields, 1993).

<u>Negative controls</u> are fusion proteins that are known not to interact with each other or to activate the reporter on their own: Plasmids pCR®2.1/p53 and pCR®2.1/VP16-CP (Invitrogen™), plasmids pBD-p53 and pAD-TRAF (Stratagene), respectively. The superfamily of tumour necrosis factor receptor-associated factors (TRAF) can transduce signals for proliferation, cell death, or NF-κB activation (Beutler and van Huffel, 1994; Bonif et al., 2006). The control pAD-TRAF expresses NF-κB activation domain and amino acids 297-503 of TRAF2, a TNFR-associated factor. TNFR is the receptor of TNF α (tumour necrosis factor α), which is a pro-inflammatory cytokine with a role in apoptosis, cell proliferation and others (Tracey and Cerami, 1993).

2.4.2 Autofluorescent control

A plasmid that codes for an autofluorescent protein was spotted on every slide to form a frame around the array in order to keep the orientation and to monitor transfection efficiency.

For this purpose a CMV driven construct pcDNA4-EGFP was generated by PCR amplification of EGFP (= enhanced green fluorescent protein) from pIRES2-EGFP (Clontech) and TA cloning into pcDNA4/HisMax TOPO (Invitrogen™). EGFP is a modified version of GFP, originally derived from the jellyfish *Aequorea Victoria*, (Bronstein et al., 1994; Prasher, 1995) and has become one of the most used reporter proteins in molecular biology (Prasher et al., 1992). The excitation maximum of EGFP is at 488 nm, the emission maximum is at 507 nm.

Alternatively, pHcRed1-N1, which expresses the BD Living Colors™ HcRed protein (Clontech), was used as a control for spot localisation and transfection efficiency. The red fluorescent protein was generated from a nonfluorescent chromaprotein isolated from the reef coral *Heteractis crispa* (Gurskaya et al., 2001). HcRed has an excitation maximum at 588 nm and an emission maximum at 618 nm.

2.4.3 Bait and prey vectors

To get bait and prey constructs, the DNA of interest has to be cloned in a corresponding vector, either by PCR amplification or restriction digest. For optimising CAPPIA, control plasmids from the kit (see section 2.4.1) were used first. Samples containing either no bait or no prey were used as additional negative controls. In these samples the pCIS-CK vector (Stratagene), which does not code for any protein, was used as "fill plasmid" to obtain equal amounts of final DNA in every sample for achieving comparable transfection conditions. Other test plasmids were bait and prey constructs coding for non-interacting proteins.

At the first plasmid pGAL/lacZ (Invitrogen™), which codes for the *E. coli* ß-D-galactosidase gene lacZ, was used as reporter. LacZ, first used for single-cell gene expression analysis in *C. elegans* in 1990 (Fire et al., 1990), was in this case detected either by immunostaining or alternatively by an enzyme-based detection protocol (see section 3.1.8).

In order to simplify the detection system and to eliminate the need for extensive manipulation of the slides, a plasmid expressing an autofluorescent reporter protein was constructed and tested. Thus GAL4-pZsGreen was created by cloning the GAL4 Upstream Activating Sequences from pGAL/lacZ (Invitrogen™) into the multicloning sites of pZsGreen1-1, a promoterless vector encoding the autofluorescent protein ZsGreen (Clontech). ZsGreen is a very bright green fluorescent protein of *Zoanthus spec*. with an excitation maximum at 496 nm and an emission maximum at 506 nm. Another tested autofluorescent reporter was GAL-Red, created from the vector pGAL/lacZ (Invitrogen™), where the lacZ gene was replaced with HcRed from vector pHcRed1-N1 (Clontech).

After optimising the conditions for CAPPIA using different control plasmids of the two used two-hybrid kits, a small library of 17 preys (Table 3) was screened for interacting protein partners of the androgen receptor ligand binding domain (AR-LBD). This library of fusion proteins in pCMV-AD was obtained from Dr. Bernhard Haendler (Schering-AG) and contains plasmids coding for genes, which are known to be associated either with AR function or with nuclear receptor function in general.

Table 3:
List of preys (samples A-Q). Constructed pAD-preys coding for genes potentially associated with nuclear receptor function.

Sample	Plasmid
A	Pea3 (full-length)
B	Pea3 N-terminal domain
C	Pea3 middle domain
D	Pea3 C-terminal domain
E	OTEX
F	Menin (aa 1-455)
G	Menin (aa 224-455)
H	Menin (aa 456-615)
I	Menin (aa 224-615)
J	Menin (aa 1-223)
K	NCoR domain
L	SMRT domain
M	Hinge region of AR
N	DBD of AR
O	NTD of AR
P	LBD of AR
Q	ALIEN domain

All plasmids were transformed into competent *E. coli* One Shot® TOP10 (Invitrogen™), selected by agar plates containing antibiotics and LB-media and purified by EndoFree Plasmid Mini Kit or EndoFree Plasmid Maxi Kit (QIAGEN). All DNA samples were dissolved in TE buffer.

2.5 Reverse transfection protocol

"Generally, if a cell can be grown in culture, it can be transfected."
(Current Protocols in Molecular Biology Online, Chapter 9,
2003 John Wiley & Sons, Inc.)

Transfection, the induction of plasmid DNA into a cell, is often done by electroporation when used cell line is non-adherent, while adherent cell lines are mostly transfected chemically. A critical factor is the necessary concentration of DNA, which depends to a great extent on the type of the cell line. Also time of incubation of the DNA in the cell culture has to be optimised for different cell lines. Thus transfection conditions need to be optimised for every cell type. In addition, in order to transfect cells with DNA immobilised as arrays, the optimisation is even more elaborate and requires the optimisation of slide surface, sample preparation and spotting procedure.

2.5.1 Methods of preparing samples

Following the protocol of Ziauddin and Sabatini there are two different methods of preparing samples for reverse transfection (Ziauddin and Sabatini, 2001). These two protocols were further optimised for application in CAPPIA experiments. In both methods gelatine powder (SIGMA©) was dissolved in MilliQ water by heating at 60°C for 15 min and sterile-filtered through a 0.45µm cellulose acetate membrane (Falcon®, Becton Dickinson). Cooled solutions can be stored at 4°C for a couple of month.

For the gelatine method the DNA is diluted with 0.2% gelatine to a final gelatine concentration of 0.17% to 0.19%. Gelatine samples can be spotted directly on slides but can also be stored at 4°C for a couple of days. After spotting spots have to dry for minimum 1 hr. Dried slides can be stored for month at 4°C in the dark.

Most of the samples in this work were prepared using an alternative, the so called lipid-DNA-method (LD-method). For this, DNA is adjusted with TE buffer to obtain equal final volumes and final concentrations for every sample. This DNA is then pre-mixed with a lipid-based transfection reagent. For CAPPIA Effectene® (QIAGEN) was used as transfection reagent. The EC-buffer of the kit (QIAGEN) containing sucrose (final concentration of 0.2 M, Invitrogen™) is mixed with DNA and Enhancer (QIAGEN). After

incubation at room temperature to let Enhancer built complex the lipid Effectene® solution is added. After another incubation 1X volume of 0.1% gelatine solution is added (final concentration of gelatine in the samples 0.05%). Prepared samples have to incubate for a minimum of 1 hr before spotting. They can be stored for a couple of days at 4°C. More suitable for high number of samples is to premix EC-buffer containing sucrose, Enhancer and Effectene® similar to the procedure of gelatine Method, and add this mix to the DNA, but this is only recommended for manual spotting (see next section).

2.5.2 Spotting

Beside tests with the microarray spotting system “VersArray ChipWriter Pro” (Bio-Rad) at the RZPD Berlin, the “sciFlexArrayer piezodispensing system S5” (Scienion AG) at the MPIMG Berlin was used as standard for automated spotting. This is based on non-contact dispensing in nanolitre volume range with piezocapillaries (Figure 3). The system has room for 18 slides arranged on 3 plate holders. Distance between dots for CAPPIA experiments was about 1.0 mm to be sure of separate transfection spots. Delivery is possible with different nozzle types (50 µm, 70 µm, and 90 µm orifice). For CAPPIA the 70 µm nozzles (generate droplets of approximately 400 pl size) were used. Larger size droplets can be generated through repetitive dispensing. Repetitive dispensing of 20 drops for one spot were found to give the best results in CAPPIA experiments (see section 3.1.5), corresponding to 8 nl sample. The frequency of dispensing is 500 Hz (500 droplets/s). All samples were spotted as triplets.

For small numbers of sample manual spotting (Figure 2) is a useful alternative to sciFlexArrayer or other automated spotting systems. For manually prepared cell arrays, the samples were spotted with a 2 µl pipette and long tips (PreCision safe seal tips® 10 µl, Biozym®) by tapping on the slide with the filled tip. Distance between the spots was about 1.5 mm. Each spot had a diameter of about 0.8 to 1.0mm and was formed by spotting about 10 nl.

After spotting, the slides were dried for minimum 1 hour but can also be stored for longer at 4°C, dark and dry. Storage is done in slide boxes placed in a Rotilabo® dryer (Roth®) filled with dry pellets and stored in a 4°C room. As alternative, storage of the slide boxes is possible at -20° or -80°C in a plastic bag filled with dry pellets.

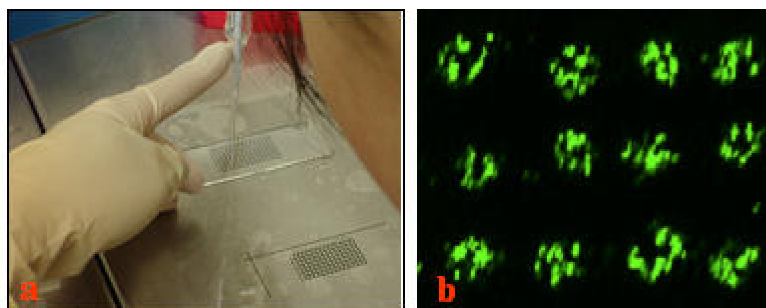

Figure 2
Manual spotting. For spotting slides manually, slides were put on top of a pattern, and long tips were used. Figure 2a shows the procedure. Distance between manual spots was about 1.5 mm, and every spot is in a range of about 10 nl. Figure 2b shows a microscope picture of a pcDNA4-EGFP manual spotted slide after transfection.

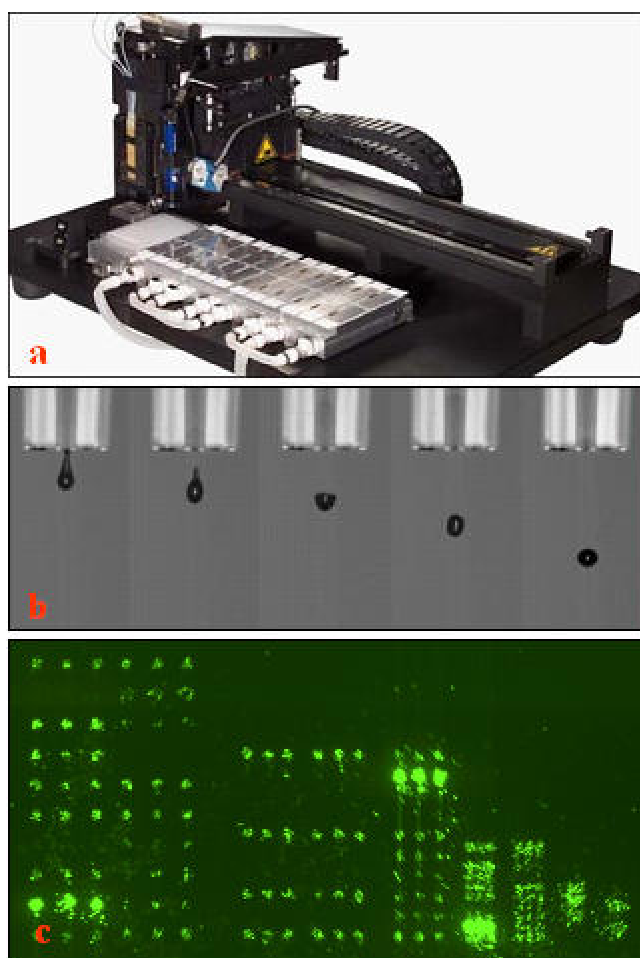

Figure 3
Automated spotting using the sciFlexArrayer. Figure 3a shows the sciFlexArrayer with its holders. Figure 3b shows droplets from a 70 µm nozzle. Different parameters such as the number of drops and spot spacings were tested to determine the best spotting protocol. Figure 3c shows an image of a transfected slide spotted with the sciFlexArrayer for testing different parameters (acquired using BIOccd camera).

2.5.3 Transfection of the cells

Slides made using the LD-method do not need to be treated with transfection reagent since the Effectene® mix was already contained in the spotting solution. Only the number of cells and volume of media required per slide has to be optimised. Slides made using the gelatine method have to be treated with transfection reagent directly before adding the cells to the slide. Per slide 191 µl of transfection mix (150 µl EC-buffer, 16 µl Enhancer and 25 µl Effectene®) was pipetted under a HybriWell™ (Whatman®, formerly Schleicher & Schuell), a plastic incubation chamber over the array on the slide. After 13 to 15 min incubation, transfection mix was removed and the suspension of the cells was added as soon as possible on top of the slide.

For experiments involved in the domains of the androgen receptor additional treatment with androgenic component R1881 is necessary to initiate reporter expression. This was done by pre-mixing R1881 with the cells before adding them to the slide to a final concentration of 10^{-8} mol. Androgenic compounds like R1881 bind and change the conformation of AR-LBD, which is necessary for full length or wild type AR to translocate from cytoplasma into the nucleus to initiate transcription (Jenster et al., 1993; Poujol et al., 2000; Torchia et al., 1998).

Although both hydroxyflutamide and flutamide are non-steroidal anti-androgens, OH-Flu is a more powerful antagonist because of its higher binding affinity for the AR (Kelce et al., 1994; Wakeling et al., 1981). OH-Flu has been used as anti-androgen in prostate cancer therapy until it was replaced by bicalutamide because of the later has less hepatotoxicity and longer half-life (Cockshott, 2004).

2.6 Fixation and staining

2.6.1 Fixation

The transfected arrays were incubated in a cell culture incubator for 48 to 72 hrs (optimum for HEK 293T around 65 hrs) with a change of the medium after two days. To stop the transfection, slides were shortly washed with PBS, fixed for 30 min with PBS solution containing sucrose (4%) and formaldehyde (3.7%) and after this washed for minimum 2 min in PBS. Slides can be stored in PBS at 4°C (dark) for up to 4 days.

After staining with DAPI (see section 2.6.5) to colour cell nuclei, a few drops of Fluoromount-G™ (SouthernBiotechnology Associoated, Inc.) were pipetted directly on top of the monolayer. Fluoromount-G™ is a non-fluorescing mounting medium to provide a semi-permanent seal for long-term storage and to reduce fluorochrome quenching during analysis by fluorescence microscopy. Slides were covered with 22 x 64 mm cover glasses (BDH), thickness No.1, which was fixed with nail polish at the edges. Covered slides can then be stored at 4°C for month while keeping fluorescent signal.

2.6.2. Immunostaining

For detection of non-autofluorescent protein expressed on the slides (e.g. for lacZ) indirect immunostaining was used. A non-marked lacZ-specific antibody binds in a first step to the antigen and a fluorescent conjugated anti-antibody binds in a second step to the first antibody.

Cells were fixed and incubated for 20 min in PBS containing 0.1 % Triton X-100 to raise the membrane permeability of the cells. After washing twice in PBS, slides were blocked for 1 hr in blocking solution containing BSA (Bovine Serum Albumin, PAA) and sodium azide (SERVA) in PBS. BSA saturates non-specific binding sites and sodium azide prevents growth of bacteria, fungi or yeast. Slides were incubated for 1 hr with the first antibody and washed 2x with PBS. After re-blocking for 1 hr with PBS/BSA, the slides were incubated for 40 min with the secondary antibody, followed by PBS washing steps.

After DAPI staining (see 2.6.5.) slides were mounted with Fluoromount-G™ and covered with cover glass as described before. Fluorescence signals of the secondary antibody could than be analysed by fluorescence microscope or standard array scanners (BIOccd).

The following primary and secondary antibodies were used:

Table 4
Primary antibodies

Name	Organism	Antigen	Dilution	Source
ab 1047	Mouse	ß-galactosidase	1:250	abcam
A-11132	Rabbit	ß-galactosidase	1:500	Molecular Probes™

Table 5
Secondary antibodies

Name	Organism	Antigen	Dilution	Source
Cy™3 115-165-146	Goat	Mouse	1:800	Jackson ImmunoRe-search
Cy™3 211-165-109	Mouse	Rabbit	1:800	Jackson ImmunoRe-search
Alkaline phosphatase A-2429	Goat	Mouse	1:50	Sigma
Alexa Fluor 488 A-11017	Goat	Mouse	1:500	Molecular Probes™
Alexa Fluor 488 A-11055	Donkey	Goat	1:500	Molecular Probes™
Alexa Fluor 594 A-11055	Donkey	Goat	1:500	Molecular Probes™

2.6.3 Alternative methods to detect LacZ

To find optimum conditions for CAPPIA, various detection methods for lacZ were tested (see section 3.1.8). Alternative to secondary antibodies Cy™3 conjugated α-mouse and α-rabbit (Jackson ImmunoResearch), signal detection by a second antibody conjugated with alkaline phosphatase was tested. Substrate for alkaline phosphatase signals were analysed by normal light microscope.

In addition enzymatic activity of lacZ was detected directly. The lacZ substrate 5-bromo-4-chloro-3-indolyl galctopyranside (X-gal) is commonly used for this. When ß-galactosidase is expressed, cells get an intensely blue colour because of the cleavage product of X-gal. However, this product is not fluorescent. An alternative for X-gal is fluorescein di-ß-D-galactopyranoside (FDG), a fluorescent ß-galactosidase substrate. Molecular Probes (Invitrogen™) offers a variant of FDG (C_{12}FDG) as part of a kit named "ImaGene Green™ C_{12}FDGlacZ Gene Expression Kit", by which substrate is supposed to enter more easily into the cells, cleaved by ß-galctosidase producing a fluorescent product. Excitation maximum of this product is at 571 nm, emission maximum is at 585 nm.

2.6.4 Normalising transfection signal

For normalising the fluorescence of samples on different slides, all signals were expressed relative to the EGFP signal on the same slide.

2.6.5 DAPI staining

On most of the slides, staining with DAPI (4`,6-diamidino-2`-phenylindole dihydrochloride, SIGMA) was carried out before covering the slides with Fluoromount-G™ (see section 2.6.1). DAPI is a fluorescent indole dye, which binds selectively to DNA and thus colours the content of the nucleus of the cell. Results are bright blue fluorescence nuclei (excitation maximum at 340 nm, emission maximum at 488 nm), which is helpful for finding the best focus during microscope scanning.

2.7 Direct transfection

2.7.1 Applications for direct transfection

To test diverse conditions, especially to test the antibody staining, direct transfection was performed. Cells in most cases were HEK 293T. 2x 10^5 cells per well were seeded out in a 6-well culture plate 1 or 2 days before transfection (depending on the cell conditions) on top of a coverslip. On transfection day, cells were washed with PBS, covered with 1ml fresh media and replaced in the incubator. The transfection mix (Effectene®, QIAGEN) including DNA at a concentration of 1-2 µg/µl per well was ready prepared and added on top of the cells. After 2 days incubation, cells were fixed and processed as described earlier following reverse transfection. For the generation of stable transfected cells, 5 x 10^5 cells were seeded out 1 or 2 days before transfection in a 60 cm^2 cell culture plate and transfected as described below.

2.7.2 Stable transfection

Three different stable transfected cell lines were made from HEK 293 cells: one stably transfected with the lacZ-reporter (=HEK 293-LZ), one with bait plasmid pBD-LBD of AR and the other with bait pBD-SMRT (=HEK 293-BD-LBD and HEK 293-BD-SMRT). Selection marker in the first case was Zeocin™ (Invitrogen™). HEK 293-BD-LBD and HEK 293-BD-SMRT were selected by geneticine disulphate G-418 (Promega), see section 2.1.2. In all cases 5 x 10^5 cells were seeded out in a 60 cm^2 cell culture plate, directly transfected with 2 µg plasmid in a transfection mix (see section 2.7.1) and treated with selection marker at a concentration pre-tested before in a 6-well-format. A few days later non-transfected cells died, and cells transfected with the plasmid expressing the selection gene started to grow in colonies. The colonies were picked and cultured separately. Different clonal cultures were testd by reverse transfection, and clones with the best results (lowest background, brightest signal) were used as the new stable transfected cell line.

2.7.3 Trans-bait transfection

Trans-bait transfection is a combination of reverse and transient transfection. Cells were handled exactly as for reverse transfection but directly before adding the cells to the slides they were mixed with 2 µg DNA complexed with transfection reagent as described in section 2.7.1. After addition of pre-mixed cells to the slide, cells were cultivated as in reverse transfections.

2.8 Analysis

2.8.1 Fluorescence microscope and BIOccd camera

Certain pigments (called fluorochroms) are able to absorb and emit light at specific wavelengths. If they are stimulated by light (=excitation) their electrons are lifted to a higher shell. When these electrons fall down to original level, they emit light (=emission). Emission wavelengths are lower than excitation wavelengths because of heat loss.

Fluorescent samples (either autofluorescent or proteins conjugated to a fluorochrome) can be analysed by fluorescence microscopy or fluorescence scanning systems. A total view of the slide was obtained with the BIOccd Image Reader (PE Applied Biosystems) using a green filter (excitation 470/30, emission 510/20) for EGFP and reporter with ZsGreen and a red filter (excitation 565/20, emission 596/14) for HcRed and reporter GAL-RED. For single spots or cells and also for scanning slides in a much higher resolution, fluorescence microscope Olymus IX 81 was used with CELL® imaging software for automated control and analysis.

2.8.2 Software for scanning and analysis

Images acquired using the BIOccd image software were handled by Axio Vision LE Rel. 4.1 (ZEISS) and converted to tagged image files (*.tiff). Images acquired using the IX 81 microscope (Olympus) were already in this format and were directly transferred to AlphaEase®FC software (Alpha Inotech) for statistical analysis.

Alternatively, GenePix® Pro 6.0 Microarray Image Analysis (Molecular Devices) was used, where the sum of means was taken as the reverence for the signal to background ratio. For normalisation, the signals were related to pcDNA4-EGFP of the slide, EGFP was set to 100% and signals were expressed relative to this value. Standard derivation then was calculated by multiplication of the calculated standard derivation with a factor obtained by 100/EGFP fluorescence signal. Calculations of means, standard deviations and creation of the graphs are made by Microsoft® Excel 2000.