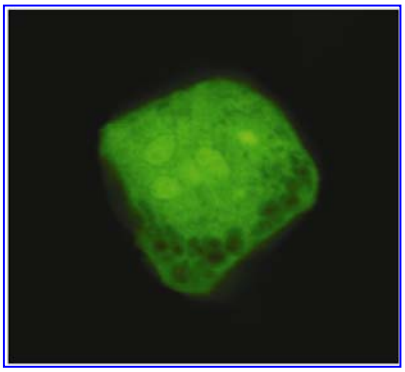
HIGH-THROUGHPUT SCREENING OF PROTEIN-PROTEIN-INTERACTIONS IN MAMMALIAN CELLS USING TRANSFECTED CELL ARRAYS



Dissertation

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Ernstzunehmende Forschung erkennt man daran, daß plötzlich zwei Probleme existieren, wo es vorher nur eines gegeben hat.

Thorstein Bunde Veblen (1857-1929)

amerikanischer Soziologe und Ökonom

TABLE OF CONTENTS

SUMMARY

The cooperation of pairs of proteins or the formation of large functional complexes of proteins is required for most if not all biological processes. Therefore, investigation of protein-protein interactions (PPI) within a cell is essential for the elucidation of biological processes and cellular networks. The two-hybrid system is the most commonly used method for PPI analyses. Hitherto, high-throughput analyses are almost exclusively performed in yeast, even when studying mammalian proteins. Putative interactions are subsequently confirmed in mammalian two-hybrid assays on a gene-by-gene basis.

The present work aimed at establishing a high-throughput, cost-effective method for analysing protein-protein-interactions directly in mammalian cells. This was achieved by combining mammalian two-hybrid system with transfected cell microarray to create the cell array-based protein-protein-interaction assay (CAPPIA). As for PCR- or oligonucleotide microarrays, the DNA samples were spotted and immobilized on glass slides in array formats. Each DNA spot contained bait and prey expression plasmids in addition to a reporter plasmid, which codes for an autofluorescent protein. After spotting the vector constructs, adherent human cells were added, creating a monolayer on the surface of the slides. Only cells growing on top of the DNA spots became transfected. In case of chimeric bait and prey protein interaction, the reporter gene was expressed, resulting in fluorescent reporter protein. Signals resulting from PPI were analyzed directly by fluorescence detection, without the need for further manipulation of the slides such as immunofluorescent staining or enzyme-based detection.

At first, production of the cell array slides and transfection conditions were optimised. Subsequently CAPPIA was shown to specifically and quantitatively detect protein-protein interactions in various mammalian cell lines. Moreover, screening of a small prey library against the human androgen receptor demonstrated that CAPPIA is well suited for the detection of hormone-dependent protein-protein-interactions. This was underscored by showing the dose-response of these interactions to androgenic compounds as well as to anti-androgenic reagents. Finally, it was shown that the possible combinatorial screens could be increased by application of slides without bait.

For this purpose microarrays consisted of spots containing one plasmid of a prey-library together with the reporter plasmid were designed. These so-called prey-reporter slides (PR-slides) were then analysed with cell lines that carried a stably or transiently expressed bait.

The high sample capacity of the cell arrays and the low reagent consumption make CAPPIA currently the most economical high-throughput detection assay for protein-protein interactions in mammalian cells. For this reason CAPPIA can become an important tool to increase the knowledge of the human interactome and thus of functional genomics.

ZUSAMMENFASSUNG

Proteinpaare bzw. größere funktionelle Proteinkomplexe sind an den meisten wenn nicht sogar an allen biologischen Vorgängen beteiligt. Aus diesem Grund ist die Erforschung von Protein-Protein-Interaktionen (PPI) essentiell für die Aufklärung von biologischen Prozessen und zellulären Netzwerken. Eine sehr leistungsstarke und häufig verwendete Methode für die Analyse von PPI ist hierbei das Zwei-Hybrid-System (two-hybrid system). Bisher werden Hochdurchsatzanalsysen (high-throughput analysis) mit diesem Verfahren aber fast ausschließlich in Hefe durchgeführt, sogar für die Erforschung von Säugerproteinen. Potentielle Interaktionen werden dann anschließend Gen für Gen mit Säuger-Zwei-Hybrid-Untersuchungen überprüft.

Die hier beschriebene Arbeit hatte die Etabliereung einer neuen, kosten-effektiven Methode für die Hochdurchsatzanalyse von PPI direkt in Säugerzellen zum Ziel. Hierfür wurde das Säuger-Zwei-Hybrid-System mit transfizierten Zell-Microanordnungen (cell microarrays) zu einem neuen Verfahren, genannt CAPPIA (cell array based protein-protein-interaction assay), kombiniert. Entsprechend der Methode von PCR- oder Oligonucleotid-Microanordnungen wurden hierbei Proben in definiertem Muster auf Objektträgern aufgetragen und dabei auf der Oberfläche fixiert. Die Proben ent-hielten vergleichbar anderen Säuger-Zwei-Hybrid-Systemen sogenannte Köder- (bait) und Opfer- (prey) Expressionsplasmide zusammen mit einem Reporterplasmid, das im vorliegenden Fall für ein autofluoreszierendes Protein codierte. Nach dem Auftragen der vektor-konstruierten Proben auf den Objektträgern wurden diese mit menschlichen, adherenten Zellen bedeckt. Diese setzten sich einschichtig auf der gesamten Oberfläche fest, aber nur die Zellen, die direkt auf den aufgetragenen DNA-Punkten (DNA spots) wuchsen, wurden durch die Proben transfiziert. Im Falle einer Interaktion der chimären Köder- und Opfer-Proteine hatte das die Expression des Reporterproteins zur Folge. Diese aus einer PPI resultierenden Signale wurden anschließend mit Hilfe von Fluoreszenznachweisen analysiert, ohne das es einer weiteren Bearbeitung der Objektträger wie Immunofluoreszenzfärbung oder Nachweisen auf enzymatischer Basis bedurfte.

Zunächst wurden die Produktion der Zellanordnungs-Objektträger und die entsprechenden Transfektionsbedingungen optimiert. Anschließend konnte nachgewiesen werden, dass CAPPIA Protein-Protein-Interaktionen in unterschiedlichen Säugerzellen spezifisch und quantitativ ermitteln kann. Darüber hinaus bestätigte das Durchsuchen einer kleinen Bibliothek von Opfer-Plasmiden, die in Beziehung zum menschlichen Androgenrezeptor standen, dass CAPPIA gut geeignet ist für den Nachweis von hormonabhängigen Protein-Protein-Interaktionen. Das wurde noch bekräftigt durch die Darstellung der Dosisabhängigkeit dieser Interaktionen - sowohl auf androgene als auch auf antiandrogene Reagenzien. Schließlich wurde demonstriert, dass die Kombinationsmöglichkeiten und damit die Effektivität des Durchsuchens gesteigert durch werden kann durch die Verwendung von Objektträgern, auf denen kein Köder-Plasmid aufgetragen ist. Jeder DNA-Fleck besteht hier nur jeweils aus einem Plasmid aus einer Opfer-Bibliothek zusammen mit dem Reporterplasmid. Diese Objektträger wurden dann mit Zellen analysiert, die stabil oder transient mit einem Köder-Plasmid transfiziert waren.

Die hohe Kapazität von Proben auf den Zellananordnungen und der geringe Verbrauch an Reagenzien machen CAPPIA zurzeit zum ökonomischsten Hochdurchsatzverfahren für den Nachweis von Protein-Protein-Interaktionen in Säugerzellen. Damit kann sich CAPPIA zu einem wichtigen Werkzeug im Bereich der menschlichen Interakteom- und funktionellen Genomforschung entwickeln.

Chapter 1

INTRODUCTION

The Human Genome Project identified around 20 000 to 30 000 protein-coding genes in the human genome – only one third of what was previously thought (2004; Lander et al., 2001; Venter et al., 2001). This means that the large increase in protein diversity is an output of alternative splicing and post-translational modifications of relatively few genes. Thus gene expression analysis alone is not sufficient for the characterisation of protein diversity in an organism. Functional genomics aims to describe genome functions and thus refers mainly to the proteomic level. This is a very broad field because of the large number of interactions, which proteins can have with different kinds of molecules - from two interaction partners to large complexes. Altogether, functional genomics is huge compared with genomic studies because even in one organism the proteome differs from cell to cell and often changes in even one cell during development, depending on biochemical interactions with genome and environment.

1.1 Microarray technology

Traditional methods in molecular biology based on the principle "one gene in one experiment" are time consuming and cost intensive regarding the amount of knowledge, which is generated. High-throughput technologies like microarrays are indispensable to conduct on genome-wide level. Microarrays are miniaturised sample carriers on special surfaces used to arrange and bind a large number of biological materials. Choudhuri defines them as a *"high-throughput assay system which utilizes spatially ordered discrete, high-density arrangement of biologically important entities immobilized on a solid platform"* (Choudhuri, 2004). "Entities" can be nucleic acid fragments, proteins, whole cells, or tissues. Using microarrays, ten thousands of genes or the whole genome can be analysed simultaneously in a single experiment. This ensures well-founded statistical comparison of the samples and the high yield of information from a particular experiment.

ELISA (enzyme-linked immunosorbent assay) (Engvall et al., 1971; Engvall and Perlmann, 1972) and the dot blotting technique for nucleic acid sequence detection (Kafatos et al., 1979) are technological forerunners of microarrays . In the late 80`s of the last century Ekins and colleagues (Ekins and Chu, 1992; Ekins et al., 1989; Ekins, 1989) designed their "microspots" and produced first microarrays with robots. Only a few years after their invention, microarrays were already well established in genetics and being used in fingerprinting and genome analysis (Hoheisel et al., 1994; Lennon and Lehrach, 1991) and the expression of sequence catalogues (Meier-Ewert et al., 1993). Researchers from Affymax Research Institute in California invented the name "DNA chips" (Fodor et al., 1991; Pease et al., 1994) and commercialised first microarrays as GeneChip® in 1996.

The most common DNA microarrays are mainly used for genome-wide quantification of gene expression or to identify genetic variations through detection of single nucleotide polymorphisms (SNPs) across populations. Also prevalent are protein microarrays, which are used to screen for the ability of the spotted proteins to bind molecules (e.g. receptors, antibodies, enzymes, hormones, or peptides). Thus this method can also be used to complement other protein-protein-interaction methods (see section 1.4). Currently, protein microarrays are often used in diagnosis of diseases by identifying a set of associated proteins.

1.2 Reverse transfection

One of the numerous applications of DNA microarrays is the method of reverse transfection first published by Ziauddin and Sabatini (Ziauddin and Sabatini, 2001). In contrast to the traditional chemical transfection (called "direct transfection" in this paper), where the DNA of interest is in solution and given together with transfection chemicals on top of cells, in reverse transfection a solution containing gelatine and the DNA of interest is spotted and dried on a glass surface. Transfection reagent is already in the samples or alternatively an additional incubation step is interposed before transfection. This microarray of spotted constructs is then covered with a layer of adherent cells, resulting in the transfection of only cells growing on top of the DNA spots and thus expression of specific proteins in spatially distinctive groups of cells. The phenotypic effect of the transfected arrays can be detected by cell-based bioassays like immunofluorescence or using autofluorescent reporter

proteins. The outstanding advantage of reverse transfection over direct transfection is its suitability for high-throughput research. One single slide can contain a set of hundreds of different samples, which are all transfected at the same time and under the same conditions. Thus reverse transfection experiments occur under a more uniform environment than single direct transfection experiments.

Reverse transfection is an appropriate method for many different domains, such as RNA interference (RNAi) research (Erfle et al., 2004; Kumar et al., 2003; Mousses et al., 2003; Vanhecke and Janitz, 2004, 2005; Wheeler et al., 2004; Wheeler et al., 2005) or for cell array-based intracellular localization screenings (Hu et al., 2005; Hu et al., 2006). It can be used to determine members of signalling pathways (Webb et al., 2003), to identify novel therapeutic targets (Mishina et al., 2004) and is also suitable for characterising proteins and their functions (Hodges et al., 2005).

1.3 Protein-protein-interaction research

Most if not all biological processes require cooperation of pairs of proteins or the formation of large functional complexes of proteins. According to estimations of Gavin and Superti-Furga there are hundreds of discrete protein complexes in eukaryotic cells, many of them containing dozens or hundreds of different proteins (Gavin and Superti-Furga, 2003). Presumably human proteins are linked with each other in 150 000 to 200 000 or more interactions (Bork et al., 2004; Figeys, 2003; Peri et al., 2003). Nearly 30 000 of them are already catalogued in the Human Protein References Database (HPRD). It is now clear that almost all proteins in a cell are part of a large protein interactome, the "*complete repertoire of interactions potentially encoded by (…) genome*" of an organism (Sanchez et al., 1999). Thus analysing protein-protein-interactions (PPI) is essential for the elucidation of biological progress, and the determination of the human interactome structure is the next big challenge after the human genome project. "*If we could map the interactions of proteins we would be able to understand protein functions*" (Figeys, 2003).

Protein-protein-interactions can be regulated in several ways. The most important is the control of the protein expression at the genomic and transcriptional level. Post-translational modifications such as phosphorylation or acetylation are also relevant, as well as the location of the protein within the cell. Some transcription factors like NF-κB are activated by decomposition of an inhibitor as a result of phosphorylation with subsequent translocation from the cytoplasm into the nucleus (Ghosh and Karin, 2002). Also important for PPI regulation is the stability of the proteins, the presence of appropriate receptors on the cell membrane, and potential ligands (see section 1.6).

1.4 Methods to detect protein-protein-interactions

It is very difficult to predict interaction partners for particular proteins, even with the knowledge of specific domain properties, like for example in the case of SH3 domain which preferentially binds to sequences containing amino acid proline (Pawson and Nash, 2003). Thus experimental approach is essential to analyse PPI, either *in vitro* or *in vivo*. The most common *in vitro* method is mass spectrometry (MS). Using this technique, not only protein pairs can be analysed but also big complexes. Interactions must be entire to be detected, MS as such is expensive and time consuming. The *in vivo* two-hybrid principle is more suitable for high-throughput PPI research and also allows mapping of interactions within a protein complex, which is very difficult when using MS. A good review of protein-protein research has been published for example by Zhu and colleagues (Zhu et al., 2003).

1.4.1 Yeast two-hybrid system

In 1989 Fields and Song demonstrated *in vivo* detection of PPI in yeast using a method they termed the yeast two-hybrid system (Fields and Song, 1989). They took advantage of the modular nature of transcriptional activators consisting of largely independent DNA-binding and activation domains (Brent and Ptashne, 1985; Keegan et al., 1986; Ptashne, 1986, 1988; Sadowski et al., 1988). Using this and the knowledge of generating hybrid activators (Brent and Ptashne, 1985), Fields and Song separated the two functional domains of the GAL4 protein of the yeast *Saccaromyces cerevisiae* and thus generated a two-part-system. In the first step a gene of interest is cloned into the "bait" vector, so that the gene is placed next to a DNA-binding domain (DBD). The bait (X) has no ability to activate the reporter gene. A second gene (or a library

of cDNAs) encoding a potential interaction partner is cloned downstream of the activation domain (AD) of the GAL4 yeast transcription factor in the "prey" vector. The prey (Y) has no ability to bind DBD-responsive elements. If the two proteins fused to the DBD and AD interact physically, they will bring the domains close together and restore a functional transcription factor that binds to the promoter of a reporter gene. The transcription of this reporter gene is activated and the related reporter protein or its catalytic activity can be detected.

The two-hybrid system, which has frequently been reviewed (Chien et al., 1991; Ito et al., 2001b; Uetz, 2002), can also be used to acquire detailed information about specific interaction domains of proteins or to determine specific amino acid residues through point mutations. The two-hybrid system is applied not only for testing interactions between known proteins but also for screening libraries for determination of new interaction partners. The first array-based two-hybrid screen of a whole proteome (*S. cerevisiae*) was published in 2000 (Ito et al., 2001a; Ito et al., 2000; Uetz et al., 2000). Recently the group of Wankers identified more than 3000 potential human PPI by using this method (Stelzl et al., 2005).

1.4.2 Limits of the yeast two-hybrid system

The yeast two-hybrid system represents one of the most efficient approaches currently available for identifying and characterising protein-protein-interactions. It is highly sensitive and detects interactions not detected by other methods (Li and Fields, 1993). However it has several drawbacks. First, the fusion proteins have to be translocated to the nucleus and must be able to fold and exist stably in yeast cells. In some cases the fusion to a transcription factor domain may occlude the site of interaction. Also interactions that need secondary modifications of the proteins, such as phosphorylation, or third interaction partners cannot be detected, which means that some true interactions stay unrecognised. These false negatives lead to up to 90% (Ito et al., 2001a) or up to 96% (Edwards et al., 2002) undetected interactions when using the yeast two-hybrid system.

On the other hand, some proteins will give false-positive signals by activating transcription without an interacting partner when fused to a DNA-binding domain (Ma and Ptashne, 1987). This may be the case for about 50% of interactions obtained from yeast two-hybrid (von Mering et al., 2002). Even with progress in this field for example from the group of Suzuki (Saito et al., 2002) it stays problematic.

Several variations of the two-hybrid system have been developed to overcome these limitations. For example in the yeast three-hybrid system (Licitra and Liu, 1996; SenGupta et al., 1996) a third partner (Z) is expressed, which is involved in interaction of bait and prey. This protein can be necessary for bridging bait and prey and can thus enable the interaction, or alternatively may prevent the interaction between them (Tirode et al., 1997; Zhang and Lautar, 1996). Other variations of the yeast two-hybrid system are the one-hybrid system (Wang and Reed, 1993), the "reverse" two-hybrid (Vidal et al., 1996) and the split-hybrid (Shih et al., 1996).

To analyse membrane proteins, the yeast two-hybrid is not suitable because of its limitation to protein partners whose interaction is assessed in the nucleus. For this demand other systems like the SRS (SOS Recruitment System) and the RRS (Ras Recruitment System) are more suitable (Aronheim, 1997; Aronheim et al., 1997). Also FRET (fluorescence resonance energy transfer) or its modification BRET (bioluminescence resonance energy transfer) can be used for this purpose (Pollok and Heim, 1999; Truong and Ikura, 2001; Xu et al., 1999). Last but not least PPI can also be detected with systems like USPS (split-ubiquitin system) (Johnsson and Varshavsky, 1994a, 1994b; Stagljar et al., 1998), MAPPIT (Mammalian Protein-Protein Interaction Trap) (Eyckerman et al., 2001), or reverse MAPPIT (Eyckerman et al., 2005).

1.4.3 Mammalian two-hybrid system

Currently, mammalian protein-protein-interactions are mainly investigated using the yeast two-hybrid system. However, in many cases post-translational procedures are essential for correct protein processing, so studying mammalian genes in yeast is problematic. These modifications can differ between organisms and even between cell types. This entails a high rate of false negatives when using a different organism from the one the genes are derived from. For example, TGF-β-induced interaction between Smad3 and c-Jun proteins is detectable only in mammalian cells and not in the yeast two-hybrid system (Feng and Derynck, 2001). Sometimes proteins can bind to an endogenous yeast protein (Luo et al., 1997), so a signal can be detected only in yeast but not in mammalian cells. This means that every potential interaction found in yeast has to be verified. Thus mammalian genes should be studied in mammalian cells, their natural environment.

Dang et al. were the first investigators to use mammalian cells instead of yeast (Dang et al., 1991). The principle of this two-hybrid assay is similar: A gene coding for a protein of interest and another coding for a potential partner are cloned to DNA-binding domain and activation domain from a transcription factor, respectively. After transfection in mammalian cells, interaction of the chimeric proteins brings the domains together and restores expression of the reporter gene.

One of the advantages of the mammalian two-hybrid system is that proteins maintain their native conformation, and additional factors necessary for the interaction of both proteins are available. This is especially relevant for proteins which interacty indirect in multi-protein complexes, for example transcription factors (Feng and Derynck, 2001). Thus, the mammalian two-hybrid-system is often used to further evaluate protein-interaction-partners found in yeast (Leonhardt et al., 1998; Luo et al., 1997) or for small-scale studies (Dixon et al., 1997). But currently mammalian two-hybrid systems involve high reagent consumption and are therefore not practical for screening PPI in mammalian cells. This is true also for variations published by various groups (Fearon et al., 1992; Fotin-Mleczek et al., 2000; Shioda et al., 2000; Vasavada et al., 1991).

The first attempt to use the mammalian two-hybrid system on a larger scale comprised transfection in 96-well or 384-well plate format (Murakami et al., 2002; Suzuki et al., 2001; Zhao et al., 2004). Automated transfection and immunostaining of mammalian cells have been established by Liebel et al. (Liebel et al., 2003). In either case, usage of microwell plate format requires automation of liquid dispensing and is characterised by high consumption of reagents.

1.5 Biology of androgen receptor

Androgens are steroid hormones in vertebrates which are essential for the development and maintenance of primary and secondary masculine characteristics (Gao et al., 2005; Lee and Chang, 2003). The most well known androgen is testosterone and its metabolite 5α-dihydrosterone (DHT). Because of its higher binding affinity (Wilbert et al., 1983) and slower rate of dissociation from the androgen receptor (Zhou et al., 1995) DHT is the more potent androgen in most target tissues.

Most androgens and androgenic components like methyltrienolone (R1881) work through receptor-mediated mechanisms (Fang et al., 2003), only a few target another site than the ligand binding domain (Yamabe et al., 2000). Anti-androgens can lead to incomplete masculinization or reduced fertility (Kelce and Wilson, 1997). They are "pure" with no other endocrine effect (e.g. flutamide) or can have gestagenic effect and in doing so act as anti-androgen (e.g. cyproterone acetate). Recently they have frequently been used to treat prostate cancer (Sharifi et al., 2005), but the precise mechanism of anti-androgenic action is still unclear (Gao et al., 2005).

The androgen receptor (AR) is a member of the nuclear receptor superfamily of ligand-dependent transcription factors (Beato et al., 1995; Evans, 1988; Mangelsdorf et al., 1995; Tsai and O'Malley, 1994), which control essential physiologic and developmental processes in humans and play an important role in prostate cancer (Heinlein and Chang, 2004). It has the common domain structure of nuclear receptors: A N-terminal activation domain (NTD, activation function 1 = AF1), a central DNA-binding domain (DBD), a hinge region and a C-terminal ligand-binding domain (LBD), usually with a second activation function (AF2) (Belandia et al., 2005; Jenster et al., 1991; Simental et al., 1991). Basis for the effects of AR is the binding of a suitable ligand. In unli-

gated status the AR is mainly localised in the cytoplasm (Jenster et al., 1993; Simental et al., 1991), associated with heat-shock proteins (HSPs), which facilitate ligand binding (Fang et al., 1996; Pratt and Toft, 1997). When a ligand binds to the AR, the confirmation changes, and the activated receptor complex enters the nucleus (Georget et al., 1997). A detailed review of this and an alternative mode of action is given by Gao et al. (Gao et al., 2005).

In 1995 Langley et al. reported interaction of NTD and LBD of AR which they inhibited with the anti-androgen hydroxyflutamide (OH-Flu) (Langley et al., 1995). Two years later Doesburg et al. described interaction of the N-terminal transactivation domain (which they named TAD) and the carboxyl-terminal domain of AR as well as its hormone dependence and the blocking by the use of different anti-androgens (Doesburg et al., 1997). They also found a weak LBD-LBD interaction and postulated intramolecular interaction between TAD and LBD of AR, in contrast to the intermolecular interaction proposal of Langley (Langley et al., 1998). However, the interaction of both domains stabilizes bound androgen and slowly its dissociation rate (He et al., 1999; He et al., 2000). Kemppainen et al. classified further AR ligands to be agonists or antagonists of this interaction and found among others OH-Flu and medroxyprogesterone acetate (MPA) (Kemppainen et al., 1999). MPA is a weak androgen *in vivo* (Bardin et al., 1983; Mowszowicz et al., 1974; Raynaud et al., 1980), but failed to induce the N/C-interaction. Its activation mechanism seams to be different from other agonists.

The nuclear receptors alter transcription through interaction with coregulators (Ikonen et al., 1997; McKenna et al., 1999). They bind to receptor complexes in the nucleus and enhance transactivation (coactivators) or reduce it (corepressors). Good overviews of coregulator functions and different modes of action are given elsewhere (Heinlein and Chang, 2002; Kumar et al., 2004; Lee and Chang, 2003; McKenna et al., 1999; Privalsky, 2004). Recently it has been found that beside pure agonists there are also partial ones, which induce a flexible state where the binding of coactivators as well as corepressors is enhanced over the unliganded state (Albers et al., 2006). In 2005 the group of Haendler characterised AR45, a variant of AR, which can either repress or stimulate the AR activity depending on levels of AR and AR45 and of cofactors such as β-catenin (Ahrens-Fath et al., 2005).

1.6 Aims of the study

The rapidly growing collection of gene sequences as a result of genome sequencing projects demands the development of systematic and high-throughput approaches for investigating protein-protein-interactions in mammalian cells. Existing techniques can deal with a limited number of genes and require automated liquid dispensing. DNA microarrays could be used to analyse genes on a genome-wide scale for relatively small costs and reagent consumption. The recently developed transfected cell arrays combine microarray technology with protein expression in mammalian cells, paving the way towards the development of new technologies for functional genomics.

The aim of this study comprised following topics:

1. Development of the high-throughput technique for screening of protein-protein-interactions in mammalian cells through the adaptation of the transfected cell array technology.
2. Establishment of proof-of-principle for this mammalian two-hybrid platform using as an example the set of genes involved in androgen receptor signalling.
3. Extension of the applicability of the established technique towards screening of a prey-library with a bait of interest in different cell lines towards investigation of cell type-specific patterns of protein-protein-interactions.

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.

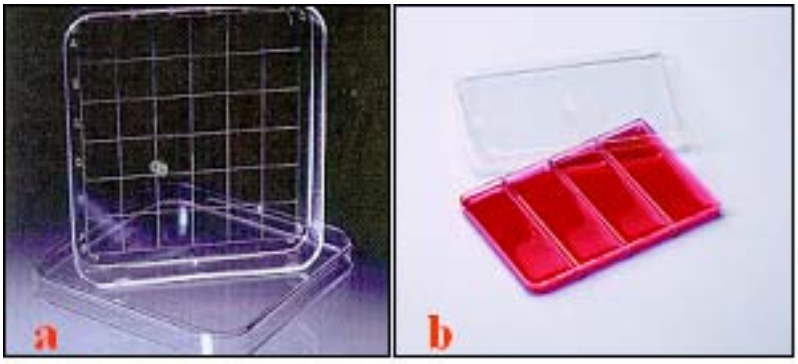

Figure 1
Suitable containers for reverse transfection. The square dish from Becton Dickinson (Figure 1a) is well-suited for 3 slides to transfect at the same time, but the Quadriperm box from Vivascience (Figure 1b) with four separate compartments is more flexible. Both containers are non-treated for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

Negative controls 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.

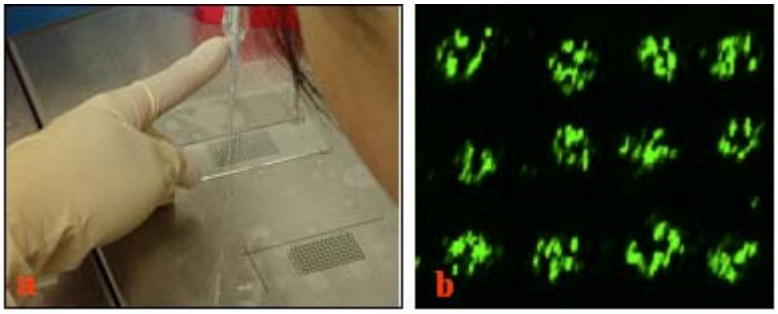

Figure 2
Manual spotting. For spotting slides manually, slides were put on top of a pattern, and long tips were used. Figure 2a shows the procedure. Distance between manual spots was about 1.5 mm, and every spot is in a range of about 10 nl. Figure 2b shows a microscope picture of a pcDNA4-EGFP manual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

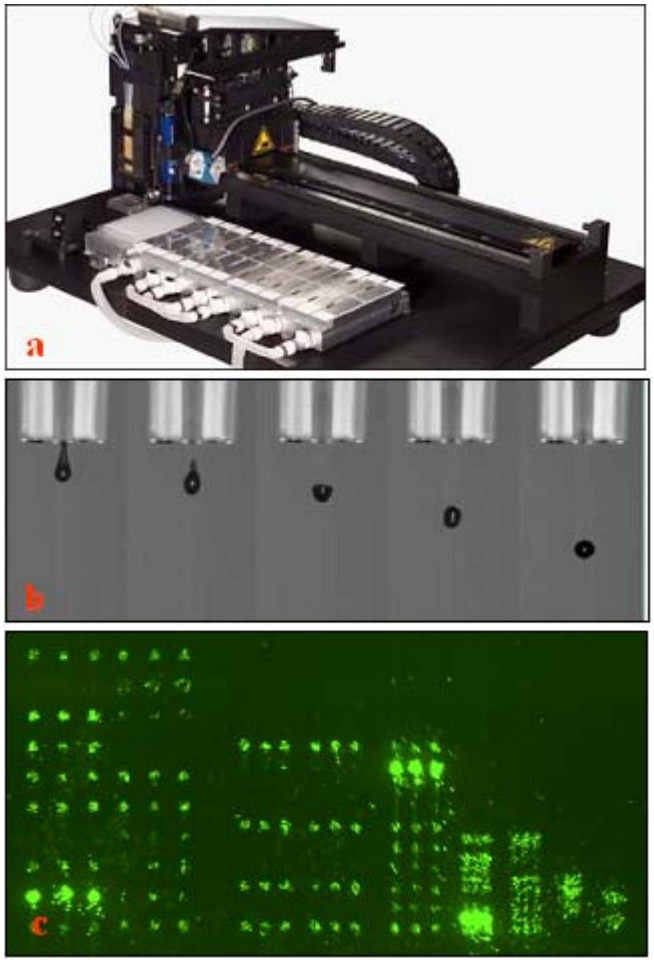

Figure 3
Automated spotting using the sciFlexArrayer. Figure 3a shows the sciFlexArrayer with its holders. Figure 3b shows droplets from a 70 µm nozzle. Different parameters such as the number of drops and spot spacings were tested to determine the best spotting protocol. Figure 3c shows an image of 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 ImmunoResearch
Cy™3 211-165-109	Mouse	Rabbit	1:800	Jackson ImmunoResearch
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.

Chapter 3

RESULTS

3.1 Optimisation

CAPPIA (= cell array based protein-protein-interaction assay) was developed as a high-throughput method suitable for detecting protein-protein-interactions directly in mammalian cells, which is fast, low in effort of time and money and easy to adapt in the lab. CAPPIA is the combination of the two-hybrid system and the principle of cell arrays, based on reverse transfection. Nanoliters volumes of solutions containing bait and prey expression plasmids and an autofluorescent reporter plasmid complexed with transfection reagent are immobilized on glass slides in array formats. When these slides are overlaid with a monolayer of living cells only those cells that grow on top of a particular spot of DNA will get transfected and will start to overexpress specific chimeric bait and prey proteins. If these two proteins can interact with each other they will transactivate the reporter, which can then be analysed in various ways. Figure 4 shows the operation of CAPPIA.

First production of the cell array slides and transfection conditions were optimised. Subsequently the specific and quantitative detection of protein-protein-interactions was tested in various mammalian cell lines as well as suitability for the detection of a hormone regulated interaction and the dose response of this interaction to androgenic compounds as well as to antagonistic reagents. To increase the flexibility, slides containing only preys and reporter plasmids and no bait (PR-slides) were used to reverse transfect cells that carried a stably or transiently expressed bait construct.

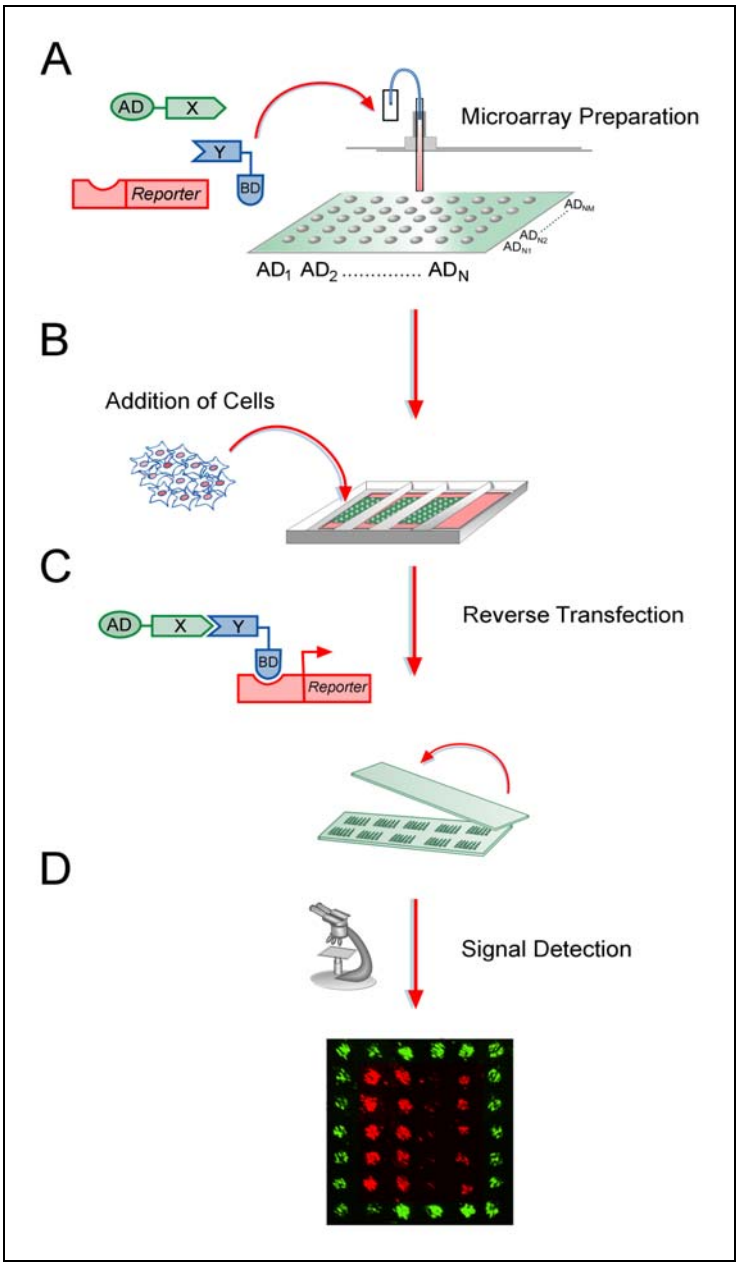

Figure 4

The CAPPIA process. A) Preparation and spotting of samples containing plasmid for bait, prey and reporter, respectively. In the bait, a gene of interest (X) is fused to an activation domain (AD). In the prey, a potential interaction partner of X (Y) is fused to a binding domain (BD). Every sample contains the same prey and the same reporter, but different bait, shown as AD1 – ADNM. After short incubation, samples can be spotted on a slide. DNA of the samples is now immobilised in separated spots. B) Adherent mammalian cells have to be added in a definite amount on top of spotted slides. They grow on these and create a monolayer. C) After a run of around 3 days while cells get transfected by spots of immobilised DNA, transfection will be stopped by fixing cells and mounting slide with a coverslip. D) In samples where prey interacts with a suitable bait plasmid, AD and BD come close together and act as transcription factor to activate expression of the autofluorescent reporter. Fluorescent signals can be analysed directly by fluorescent microscope or scanning systems (red dots). In case of non-interaction of bait and prey, the area of spot is dark because of no fluorescent reporter protein. A frame of autofluorescent plasmid pcDNA4-EGFP (green dots) helps orientation on slide.

3.1.1 Slide surface

Cell array performance strongly depends on the quality of the microarray surface. So testing slide surfaces was one of the earliest steps when developing CAPPIA. DNA has to be well attached to the slide surface and at the same time has to be easily taken up by the cells during transfection. Also the cells have to stick well to the slide surface to create a continuous monolayer. GAP™ coated slides, suggested by the group of Sabatini (Ziauddin and Sabatini, 2001), were tested and compared with various commercial and self-made slides (see section 2.2, Table 1).

Different slide types (see following sections for details) were used for spotting of pcDNA4-EGFP for expression of autofluorescent protein and pGAL/LacZ for testing the stability of the cell monolayer during the extensive immunostaining treatment. Additional pBD-p53 + pAD-SV40T as positive control and pBD-p53 + pAD-TRAF as negative control were spotted together with reporter plasmid GAL4-pZsGreen, respectively. Before fixing the transfected cells, monolayer of each slide was inspected visually. After fixing and covering with coverglass, monolayer was again inspected by eye and additionally by microscope for the presence of fluorescent signals and monolayer quality.

Poly-L-lysine slides became a standard for many different microarray experiments. Both self-made and commercially available poly-L-lysine slides were tested. Signals of expressed proteins were mostly well detectable. The cell monolayers were less preserved in self-made slides than with commercial slides (Figure 6). In order to improve performance, self-made poly-L-lysine slides were additionally covered with silane (a silicon derivative used as adhesive agent, linker or for water removal). Silane containing an amino group is often used to attach proteins and DNA to glass surfaces. Beside weak signals for a few spots nearly no fluorescence could be detected using this type of slide.

GAP™ slides (coated with gamma amino propyl silane) (Corning) provided satisfactory results concerning all parameters. The monolayers were often nearly faultless. The fluorescent signals were in nearly all cases of good intensity (Figure 6). Even more satisfactory concerning all parameters were self-made slides covered with poly-L-lysine and VECTABOND™ Reagent from Vector Labs (VPL slides). VECTABOND™ enhanced the adherence of cells to

the glass surface and thus improved performance of poly-L-lysine as a slide-coating agent. This is due to the presence of the positive loaded amine groups for initial ionic attachment of the negatively charged phosphate groups in the DNA backbone. Slides coated only with VECTABOND™ Reagent gave very poor results for monolayer quality and expression efficiency of the spotted DNA In contrast, VECTABOND™ with poly-L-lysine turned out to be as good as or even better than GAP™ slides. The cell monolayer wqas much better than with poly-L-lysine slides (Figure 5) and remained stable even after fixing, immunostaining or other chemical procedures. The fluorescence signals derived from the pcDNA4-EGFP transfected cells were clear and bright (Figure 6). Intensity of the fluorescence from the secondary antibody was lower on all types of slides, but signals were still well recognisable when using fluorescence microscope and BIOccd camera.

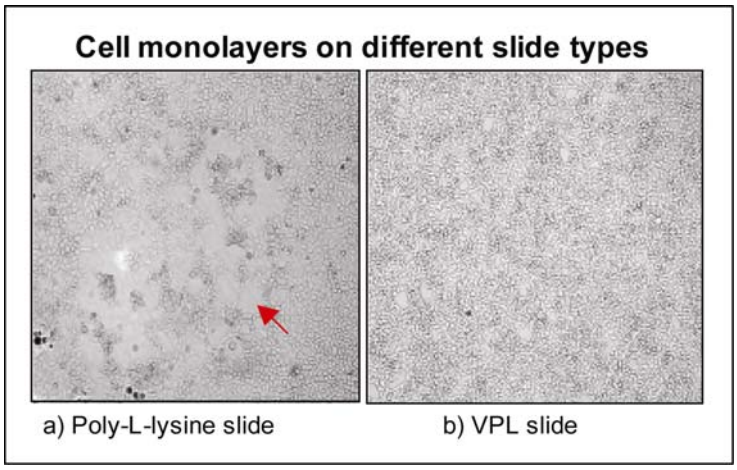

Figure 5

Monolayers of transfected HEK 293T cells on different slide types. Poly-L-lysine slide (from TeleChem`s ArrayItTM) (Figure 5a) featured more gaps (red arrow) in the monolayer than a self-made VPL slide (Figure 5b). Image was acquired using microscope.

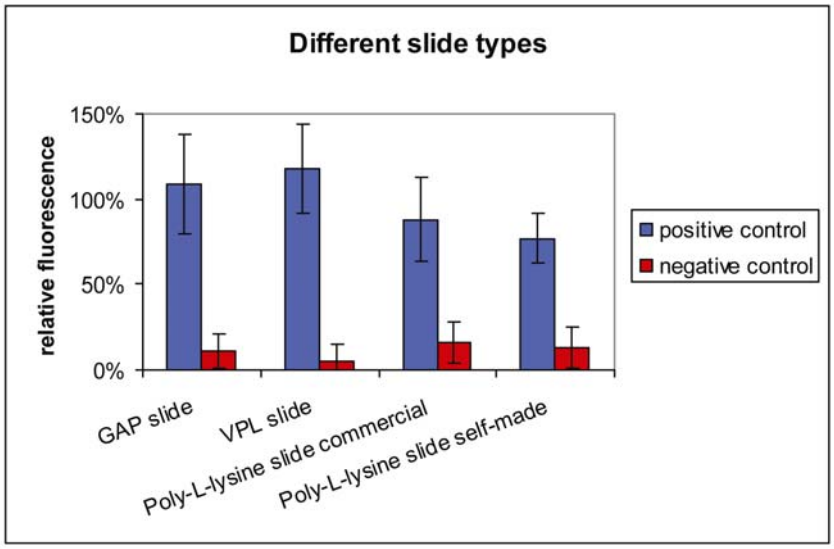

Figure 6
Fluorescent signals from detection of expressed proteins on different slide types. Graph of relative fluorescence of positive and negative controls on GAP™ slides, VPL slides and two types of poly-L-lysine slides (commercial and self-made). Spotted plasmids were pBD-p53 + pAD-SV40T as positive and pBD-p53 + pAD-TRAF as negative control with reporter GAL4-pZsGreen, respectively. Autofluorescent protein EGFP was used as reference fluorescence signal.

Figure 5 and Figure 6 show cell monolayers and fluorescence signals on manually spotted slides. Results for GAP coated slides, poly-L-lysine slides and VPL slides with automated spotting were comparable. While poly-L-lysine slides performed worse, GAP™ and VPL slides were similar.

3.1.2 Reverse transfection on coverslips

In order to obtain a continuous monolayer, approximately 3.5 x 10^6 HEK 293T cells were necessary to cover a regular slide for reverse transfection (for other cell types this number can vary, see section 2.1.2). This is a potential limitation for application of cell types, which are difficult to proliferate in culture, e.g. primary cells. Thus the use of VPL coverslips instead of slides was tested, using the same preparation protocol (see section 2.2). It turned out that the addition of 4 x 10^5 HEK 293T cells was sufficient to obtain a good monolayer. As with regular slides, best results were obtained by culturing cells for 48 to 72 hrs after transfection.

Overall, the reverse transfection of cells on VPL coverslips was possible, but fluorescence signal intensity remained lower then for regular slides, and the cell monolayer was frequently disturbed. Because CAPPIA was envisaged as a high-throughput tool and also fibroblasts were successfully reverse transfected on slide format (see section 3.1.9), reverse transfection on coverslips was not further pursued.

For small areas the Lab-Tek™ Chamber Slide™ System (Nunc™) (Figure 7) proved to be more practical and easier to handle than the coverslips, even though their surface was not coated with VPL. The spotting protocol as well as transfection length was similar to that for regular slides. When using the 4-well chamber slide, only small numbers of cells were necessary (approximately $4x10^5$ HEK 293T per well), making this system suitable for slow growing cell lines. Furthermore it appears to be practical for comparing the influence of different components in the media (e.g. androgens or anti-androgens) and thus for example to test hormone-dependency of an interaction (see section 3.2.2).

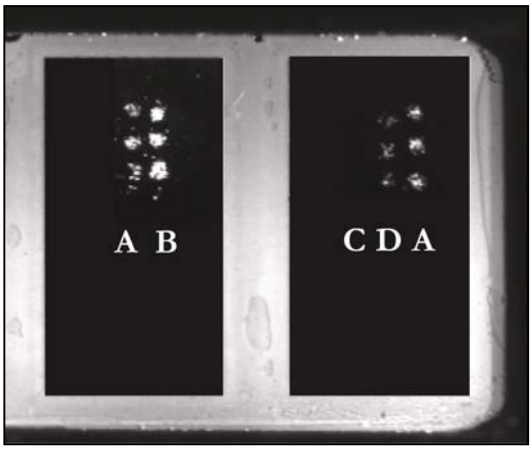

Figure 7
Reverse transfection on a 4-well Lab-Tek™ Chamber Slide™. Spotted plasmids on two wells were pBD-NFkB (B), pBD-p53 + pAD-TRAF as negative control (C) and pBD-p53 + pAD-SV40T as positive control (D) together with GAL4-pZsGreen as reporter, respectively. Autofluorescent control plasmid was pcDNA4-EGFP (A). The small areas make this system useful for slow growing cell lines. Image was acquired using BIOccd.

3.1.3 Sample preparation

In order to reverse transfect mammalian cells, samples containing the DNA and gelatine for temporary attachment to the glass surface have to be prepared. The concentrations of these two components are crucial for efficient transfection.

Concentration of DNA
Samples of pcDNA4-EGFP prepared using gelatine method were spotted at various concentrations between 5 ng/µl and 150 ng/µl. For reverse transfection with single plasmid per sample, DNA concentrations between 40ng/µl and 70 ng/µl gave the strongest fluorescent signals for pcDNA4-EGFP (Figure 10) and pGAL/LacZ. A concentration of 150 ng/µl was cytotoxic in all tests.

In CAPPIA experiments three plasmids have to be transfected at the same time. This "triple-transfection" with bait, prey and reporter plasmid requires extensive optimisation of the transfection protocol. The total amount of DNA has to be considered as well as the concentration of every plasmid. Concentrations were tested for each plasmid in the range from 10 ng/µl, in intervals of 10, up to 70 ng/µl in different ratios of bait to prey to reporter (1:1:1 / 1:1:2 / 2:2:1). A concentration of 50 ng/µl for each plasmid at a ratio of 1:1:1 represents a total DNA concentration of 150 ng/µl in the sample.

For samples prepared with the gelatine method the best concentrations were 30 ng/µl for bait and prey respectively, and 50 ng/µl for reporter plasmid. The total amount of DNA should not exceed 110 ng/µl. With DNA concentrations over this limit the signal intensity of the fluorescence was clearly lower. Thus, the ratio of plasmids is a compromise between the need of sufficient amount of each plasmid and the danger of cytotoxicity if the total DNA concentration is too high. In this context it turned out that a higher concentration of the reporter was more important than the concentrations of bait and prey.

However, this was not necessarily valid for LD-prepared samples. Using this method, different ratios of bait to prey to reporter (1:1:1 / 1:1:2 / 2:2:1) were compared with final concentrations of DNA ranging from 20 ng/µl, at intervals of 10, up to 80 ng/µl. Prepared 50 µl sample solution with final concentration of around 50 ng/µl contained 800 ng of each plasmid (bait, prey and reporter). The best fluorescent signals were found with DNA concentration of

approximately 50 ng/µl, but results fell off sharply above 60 ng/µl (Figure 8). Increasing the reporter concentration did not further improve the quality of the fluorescent signal.

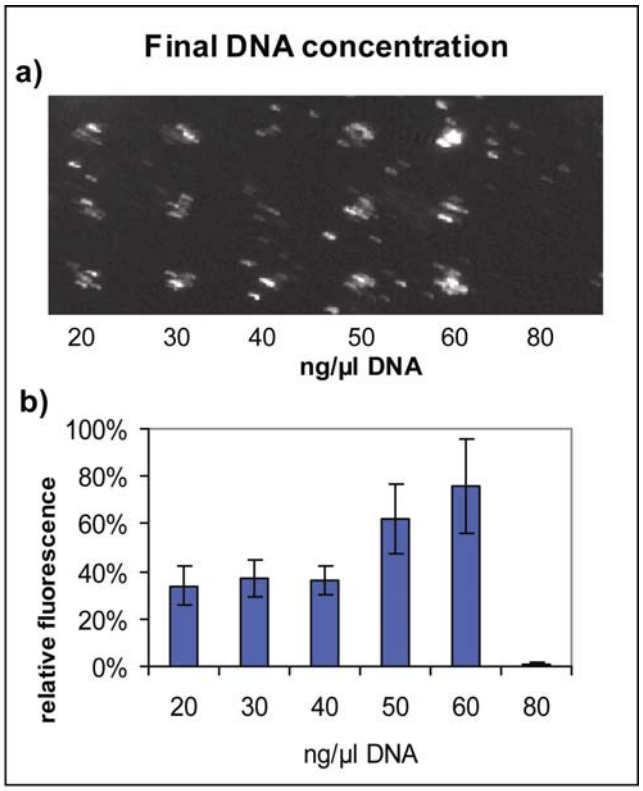

Figure 8

Different final concentrations of DNA in samples prepared by LD-method with three plasmids ("triple-transfection"). Tested plasmids were pBD-p53 together with interacting partner pAD-SV40T and GAL4-pZsGreen as reporter. The best fluorescent signals were obtained with final DNA concentrations between 50 and 60 ng/µl. Plasmid for auto-fluorescent protein EGFP was spotted as reference value for fluorescence. Figure 8a shows image acquired using BIOccd camera. Figure 8b shows quantification of fluorescent signals.

Gelatine concentration

The presence of gelatine in the DNA solution is essential for temporary character of the DNA immobilisation to the glass surface. The final gelatine concentration in the spotting solution is also relevant for automated spotting procedure, since the robot will not work properly if the viscosity of the solution is too high. For gelatine method, final concentrations from 0.1% to 0.25% were evaluated. At first, to compare various solvents 0.2% gelatine solutions were prepared with MilliQ water, with Tris and with TE buffer, respectively.

No differences were detected, so MilliQ water was used in further experiments. It turned out that for samples prepared with the gelatine method the final concentration has to be between 0.17% and 0.19% for the best fluorescent signals, especially when an automated system is used for spotting. Higher gelatine concentrations resulted in lower fluorescence and spot smearing.

For LD-method, concentrations were tested from 0.025%, to 1.5% (Figure 9). Gelatine solutions with final concentrations of 1.0% and higher were difficult to handle because of the viscosity, and concentrations of 0.5% and higher were problematic to use after storage at 4°C. The best concentration for preparation of LD-samples was 0.2% gelatine solution, with a final concentration of 0.1% in the sample solution. Sterile filtered and aliquoted gelatine solution could be stored for at least 3 months at 4°C.

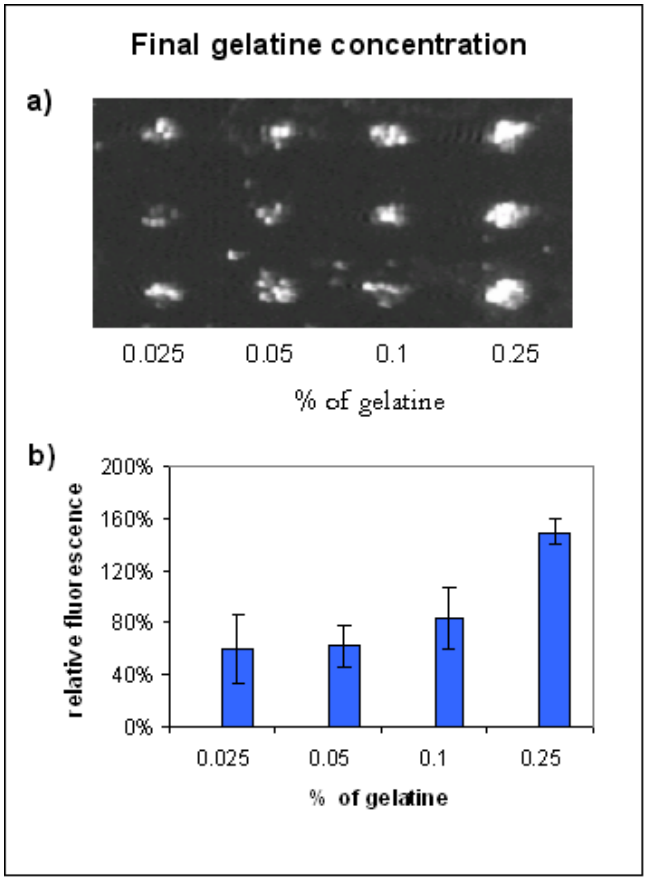

Figure 9
Different concentrations of gelatine in samples prepared by LD-method. Tested plasmids were pBD-p53 together with interacting partner pAD-SV40T and GAL4-pZsGreen as reporter. PcDNA4-EGFP was spotted as reference value for fluorescence Best fluorescent signals of the reporter protein were found with final concentrations of 0.25%. Because of an easier handling, final concentration of 0.1% was chosen as standard for CAPPIA experiments. Figure 9a: BIOccd camera image. Figure 9b: Fluorescence quantification.

Comparison of gelatine- and LD-method

The two possible ways to prepare samples for reverse transfection, the gelatine method and the LD-method (Ziauddin and Sabatini, 2001), were compared by spotting pcDNA4-EGFP on separated regions of the same slides as well as on different slides. The results were the same in both cases.

On manually spotted slides, the gelatine method turned out to give good and reliable fluorescent signals in CAPPIA experiments, but even after optimisation these were always lower than for samples prepared by the LD-method (Figure 10). On automated spotted slides made by the VersArray ChipWriter Pro (Bio Rad) (see section 2.5.2) only samples prepared by gelatine method provided good fluorescence signals. Even after a lot of optimisation steps it was not possible to amplify the signal with LD-method samples to an acceptable degree. In contrast, LD-samples spotted with the sciFlexArrayer piezodispenser S5 (Scienion AG) (see section 2.5.2, too) were distinct and bright, of much better quality than samples prepared by the gelatine method. This is comparable with the results of the manual spotting. Thus, LD-samples, spotted either manually or robotically with the sciFlexArrayer, were used in further experiments (see section 3.1.5).

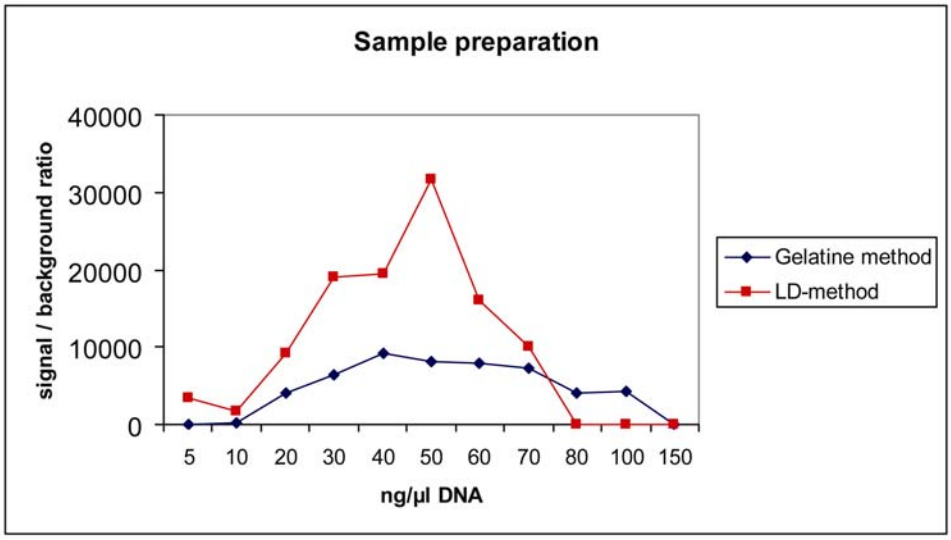

Figure 10

Different DNA concentrations in samples prepared by gelatine method compared to samples prepared by LD-method. The pcDNA4-EGFP plasmid was spotted manually. Fluorescent signals of LD-prepared samples were brighter at each concentration than signals with gelatine method, but toxicity began at lower DNA concentrations (80 ng/µl) The gelatine method samples still gave signals at 100 ng/µl. Best results for LD-method were found for 30 to 60 ng/µl DNA, with a peak at 50 ng/µl. For samples prepared with the gelatine method best results were found between 40 and 70 ng/µl.

3.1.4 Conversion to fluorescent reporter

At the beginning of the project, pGAL/lacZ was used as reporter, detected by immunostaining. Later the system was adapted to autofluorescent-based GAL4 driven reporters. The GAL4-pZsGreen plasmid construct expresses the green protein ZsGreen. Another reporter plasmid (GAL4-Red) encodes for red protein HcRed. Tests showed that both reporters work efficiently. For most of the experiments GAL4-pZsGreen was chosen because of the bright fluorescence signals of ZsGreen, in a comparable range or even brighter than EGFP (Figure 11). In Figure 12 fluorescence of HcRed and ZsGreen are compared for signals of positive control pBD-p53 + pAD-SV40T and negative control pBD-p53 + pAD-TRAF.

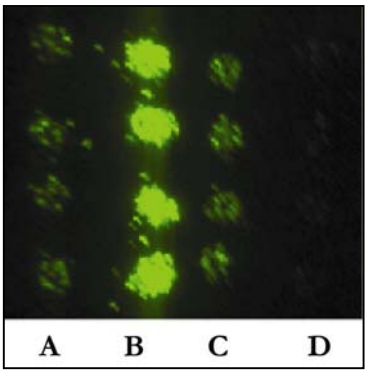

Figure 11
Expression of fluorescent ZsGreen protein in comparison with EGFP. Reporter plasmid GAL4-pZsGreen was co-transfected with pBD-NF-κB (B), pBD-p53 + pAD-SV40T (C) and pBD-p53 + non-interacting partner pAD-TRAF, where no ZsGreen was expressed (D). PcDNA4-EGFP was spotted nearby (A) as autofluorescent control. Fluorescent signals of expressed reporter protein ZsGreen was bright and in the case of co-transfection with pBD-p53 + pAD-SV40T in a comparable range with autofluorescent EGFP. For pBD-NF-κB transfection the fluorescent signal was even much brighter than EGFP. Image acquired using BIOccd.

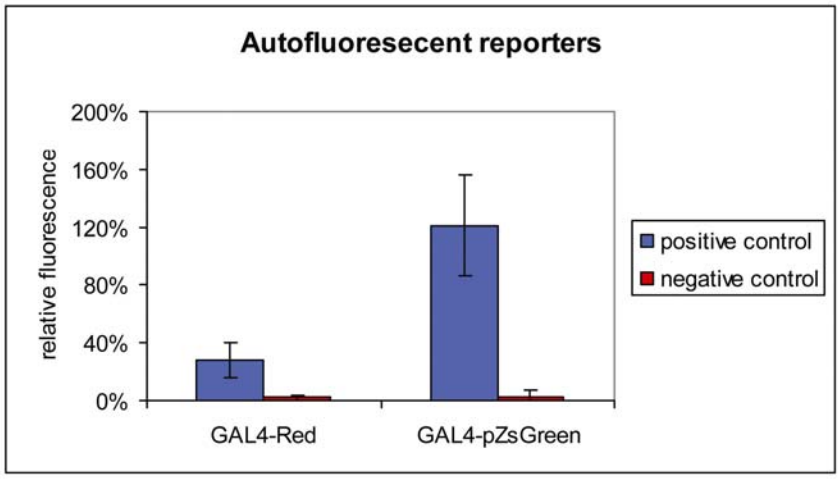

Figure 12
Signals of red reporter GAL4-Red and green reporter GAL4-pZsGreen. Expression of reporter proteins was in consequence of transfecting cells with pBD-p53 + pAD-SV40T as positive control. Nearly no reporter was expressed when cells were transfected with non-interacting pBD-p53 and pAD-TRAF as negative control. Fluorescence is shown at the ratio of corresponding autofluorescent controls: HcRed for GAL4-Red and EGFP for GAL4-pZsGreen, which turned out to be more suitable than the red reporter construct.

3.1.5 Spotting

Once the samples are prepared they have to be spotted on the slides to create microarrays for reverse transfection. Spots have to be clearly separated from each other and fixed well on the surface. On the other hand cells have to be able to take up the DNA from the spots.

Manual spotting

Manual spotting turned out to be very practicable for small numbers of samples and for testing parameters to optimise the protocol. A pattern of about 1.5 x 1.5 mm, on which slides were fixed during the manual spotting, was designed to get regularly arranged samples. Tips of different size and length and different numbers of tapping on the surface with the tip (from 1x to 4x) were tested. Best results were found when using the PreCision safe seal tips® 10 µl (Biozym®) and tap once on top of the slide (see section 2.5.3). Apart from the risk of not hitting exactly the top of the first spot, tapping for more than one time only made spots bigger, but did not enhance the fluorescent signal. In some cases the signals were even worse.

For better orientation during analysis the spotting area was marked with black marker pen (permanent Lumocolor, thickness F, Staedtler®). Red and blue markers of the same source detached and turned out to be toxic for HEK 293T, HeLa and probably other cell types.

Automated spotting

Two different robot systems were evaluated to find the best protocol for automated slide spotting. At the beginning tests were carried out under the direction of Dr. Wagner with the robot system for microarray spotting VersArray ChipWriter Pro (Bio-Rad) at the RZPD Berlin. The spotting pin was one important factor in the optimisation phase. Stealth Microarray Spotting Pins of the sizes SMP3, SMP4, SMP4B and SMP9 (TeleChem international) were compared (Table 6). The pins have flat tips and uptake channels to form a thin layer of sample at the end, allowing a gentle contact with the surface. Distances between the spots were 500 µm.

Table. 6:
Different pins from TeleChem tested for CAPPIA experiments.

Catalog number	Spot diameter (µm)	Uptake volume (µl)	Delivery volume (nl)	Number of spots per loading
SMP3	100	0.25	0.7	200
SMP4	135	0.25	1.1	185
SMP4B	145	0.60	1.4	470
SMP9	300	0.25	3.3	110

As presented in Figure 13, usage of the SMP4 pin gave the best fluorescent signals of EGFP in CAPPIA experiments. While SMP3 was barely worse than SMP4 – in microscope check as well as in analysis of the fluorescent EGFP signals after transfection – the SMP4B pin seems to be better than the SMP4 in microscope check after spotting, but turned out to be worse in a comparison of the fluorescent signals of EGFP. The mean intensity of the signals was lower by a factor of four than with usage of SMP4. Then another pin size (SMP9) was tested, but it did not improve results obtained with the SMP4 pin. Thus SMP4 was selected as standard pin for the subsequent robot experiments.

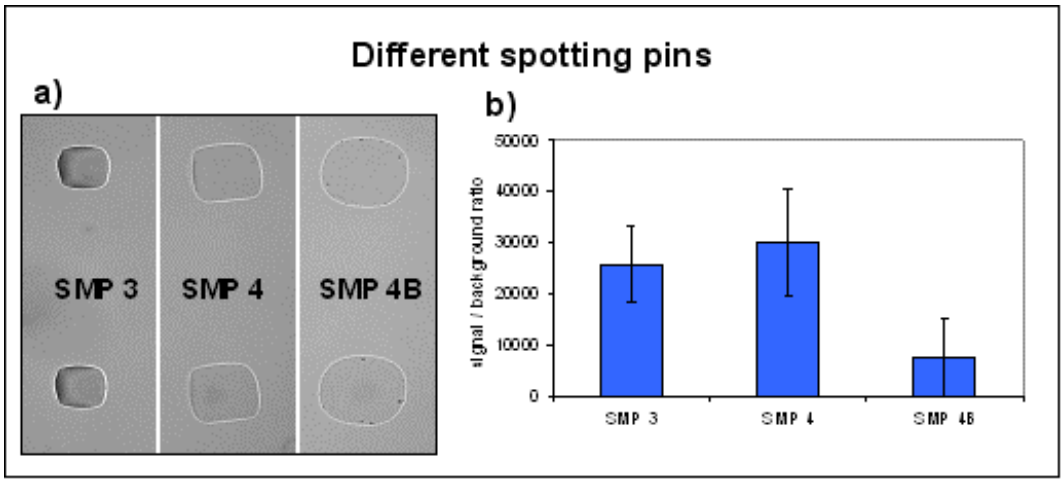

Figure 13
Comparison of different spotting pins used for automated spotting using pcDNA4-EGFP (gelatine method) as a DNA sample. First check was done before transfection with the microscope (Figure 13a). Here the SMP4B pin seems to be best. But signal intensity of EGFP fluorescence after transfection (corresponding graph, Figure 13b) was much better with the SMP4 pin and worst with SMP4B.

For automated spotting, the gelatine method was tested as well as LD-prepared samples containing pcDNA4-EGFP and DsRed. Final concentrations were 30 ng/µl and 60 ng/µl, respectively. Printed spots were checked before transfection by eye or microscope. More than 99% of the spots were visable after some general optimisation steps with the robot. It turned out that for LD-samples touching the pin to the slide ten times longer delivers better transfection results (500 msec touching time for LD-samples and 50 msec touching time for samples prepared with the gelatine method). But even with this and other changes, like the distance between pin and surface, the results of LD-samples were always worse than gelatine-samples.

However, amplification of the fluorescent signal of gelatine-samples could be achieved by repetitive spotting, by touching on the same spot for twice to five times with drying time in between. Microscope checks after spotting showed that the robot spotted very exactly on top of the previous spot. Four times spotting was found to be best (Figure 14).

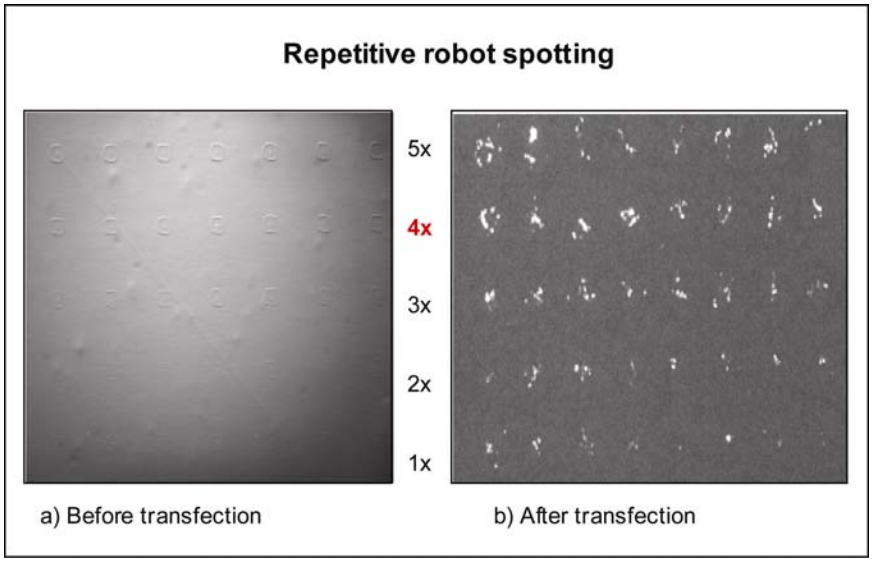

Figure 14
Repetitive robot spotting for amplification of fluorescent signal. Sample of pcDNA4-EGFP was prepared by gelatine method and spotted on slide for 1 up to 5 times on the same area with drying time in between. Before transfection, spots were checked by microscope (Figure 14a). After transfection fluorescent signal of EGFP was used for analysis (Figure 14b). The microscope check revealed repetitive spotting for four times to give the best results. Five times spotting made the signals intensity worse.

The sciFlexArrayer piezodispenser S5 (Scienion AG) was tested as another robotic system for spotting CAPPIA samples. It is based on non-contact dispensing in nanolitre volume range by using piezocapillaries, which work with system fluid (the sample solution is aspirated in contact to the system fluid). A distance between the dots of 1.0 mm was adequate to be sure of separate transfection spots and the chosen 70 µm nozzle, which generated droplets of approximately 400 pl, gave good results. To get larger spots, repetitive dispensing was tested (Figure 15). Different numbers of droplets (5 to 40) were compared before and after transfection. While the microscope check of fluorescent nucleotides in the spotted samples before transfection shows that 40 droplets should be selected for further tests, best fluorescent transfection results were found with not more than 20 droplets (total volume of 8 nl). The main advantage of the system is its flexibility, which makes it possible to test a lot of parameters in parallel and thus makes optimisation fast and efficient. Therefore, the sciFlexArrayer was chosen as standard automated spotting system in further experiments. After only a few optimisation steps, good and reliable results could be achieved, especially with LD-samples.

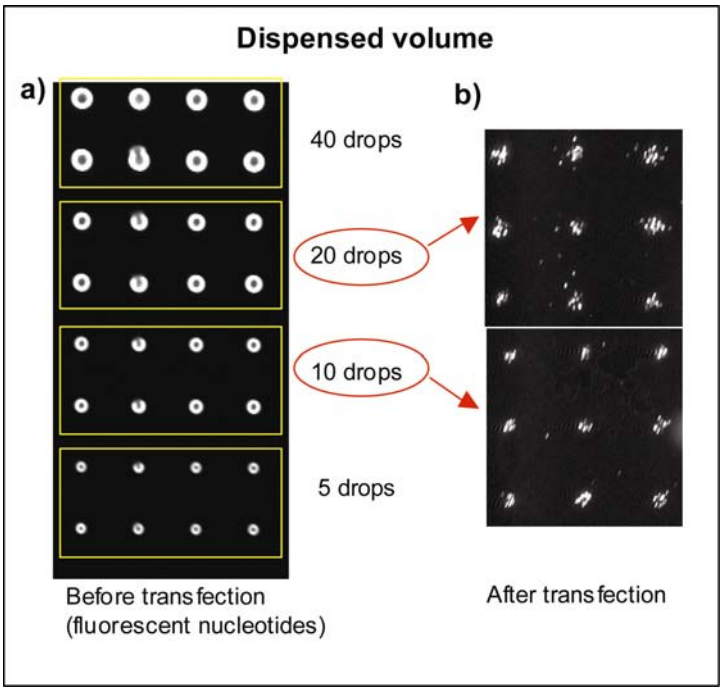

Figure 15

Dispensing of different sample volumes using the sciFlexArrayer piezodispenser S5. 5 to 40 droplets per spot were tested. Figure 15a shows microscope image of spotted fluorescent nucleotides before transfection. Figure 15b shows fluorescence of EGFP with 10 drops compared to 20 drops per spot after transfection, acquired using BIOccd camera. While best spotting results before transfection were found with 40 drops per spot, the check up after transfection showed 20 drops per spot to be better. 10 drops per spot caused less EGFP fluorescence and with more than 20 drops signals decreased.

3.1.6 Treatment after spotting

Storage

If not immediately used, spotted slides (prepared by either gelatine- or LD-method) have to be stored in darkness and dry conditions. They were stored at 4°C in a dryer, or alternatively at –20° or at –80°C for longer periods in a plastic bag filled with drying pearls. In order to test the stability of these slides, they were used for reverse transfection experiments more than 4 month after spotting and storage at 4°C (Figure 16). Cells were successfully transfected with spotted pcDNA4-EGFP. Signal of expressed fluorescent protein EGFP was bright and as clear as with freshly prepared slides.

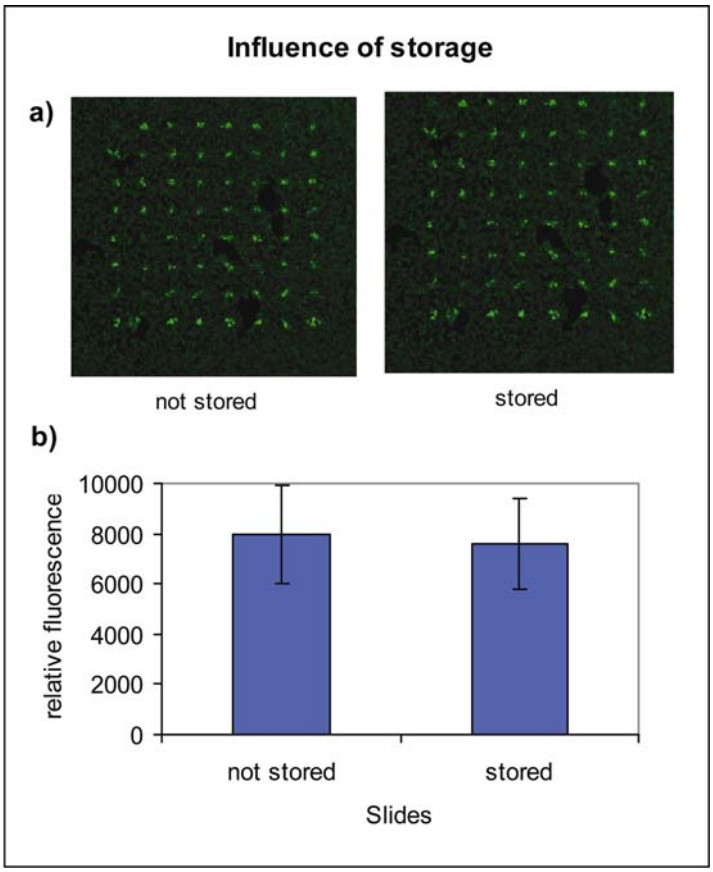

Figure 16

Storing slides does not lessen fluorescent signals on a robotically spotted slide. Figure 16a shows a slide stored for 4 months after spotting in darkness, dry and at 4°C. Transfection of spotted pcDNA4-EGFP, prepared by LD-method, was successful even after this long storing time, as shown by fluorescence of EGFP. Image acquired using BIOccd camera. Figure 16b shows the graph of a robot spotted slide used for reverse transfection directly after spotting (not stored) compared to the slide stored for 4 month before transfection (stored). Fluorescent signals of both were in the same range.

Rehydration of spotted slides

To test if rehydration of the slides after spotting would result in better fluorescent signals, slides were rehydrated for 2 min and then baked for 10 sec at 150°C. The fluorescent spots were clearly worse than without rehydration. When rehydrated for 2 min and than air-dried, the fluorescent signal intensity was in the same range as without rehydration (Figure 17). Therefore rehydratation after spotting was not performed in further studies.

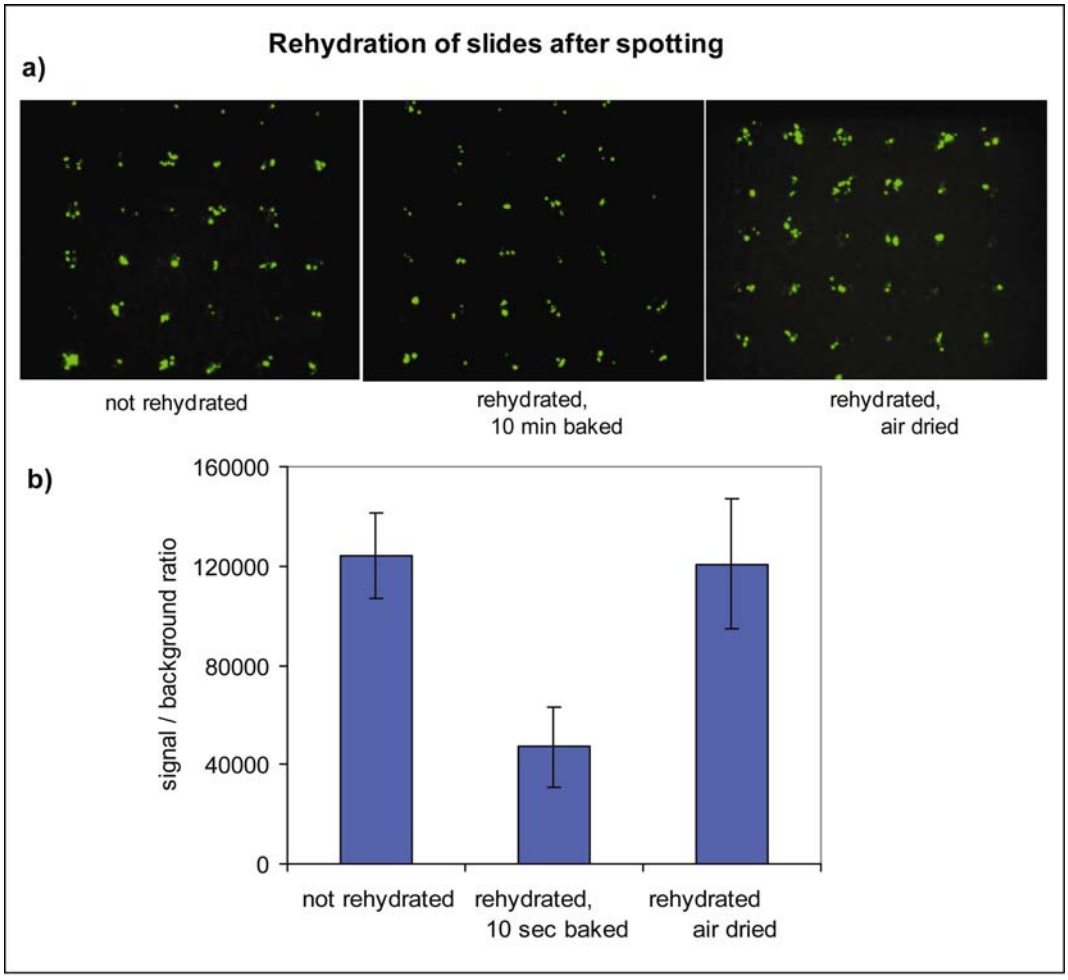

Figure 17

Effect of rehydration on robotically spotted slides before transfection. Fluorescent signals on the not-rehydrated control slide were much brighter than signals on the rehydrated one baked for 10 seconds, but in the same range as the air-dried rehydrated slide. Figure 17a: Images of the slides under microscope. Figure 17b: Quantification of fluorescent signal to background ratio of EGFP fluorescence.

3.1.7 Transfection reagents

For reverse transfection the transfection reagent has to be added to DNA before spotting (LD-method) or after (gelatine method). For samples prepared by gelatine method, transfection mix was incubated in a HybriWell™ for 10 to 30 min, with best results after 13-15 min incubation. PEI (polyethylenimine), known to facilitate transfection of mammalian cells, was tested as a more economical alternative to Effectene®. Different times of incubation from 10 to 45 min were evaluated as well as different concentrations and ratios of EC-buffer and Enhancer solution, which are components of the Effectene® transfection reagent kit (QIAGEN).

PEI was found to be best when used similarly to Effectene procedure (in mixture with EC-buffer and Enhancer) but in a double amount and with 15 min incubation in a HybriWell™. With this protocol, cells were transfected, but the background was higher than with Effectene® and the fluorescent signal intensity was lower (Figure 18). Another transfection reagent tested was TransMessenger Transfection Reagent (QIAGEN), designed for transfection of RNA. As shown in Figure 18 it did not give better results than Effectene®. Therefore the Effectene® was chosen as standard transfection reagent in further experiments.

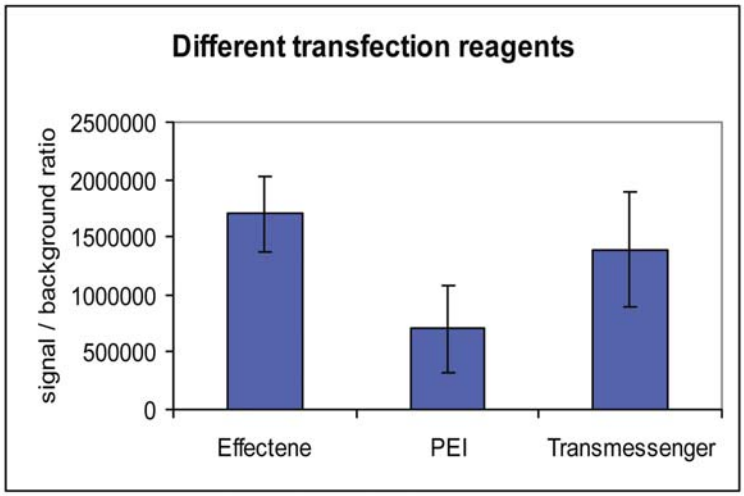

Figure 18
Comparison of different transfection reagents for reverse transfection. Graph of ratio of EGFP signals to background of non-transfected cells was best with Effectene® and a little bit lower but in the same range with TransMessenger Transfection Reagent. Reverse transfection with PEI was successful when used similarly to Effectene® (with EC-Buffer and Enhancer solution). It resulted however in clearly lower range of fluorescent signal to background ration than other tested transfection reagents.

In order to simplify the transfection protocol, which is advisable especially for high-throughput research, it was tested if transfection reagent could be premixed with the media instead of incubated in a HybriWell™. However, virtually no fluorescence signal was detected, and the cell monolayer was perforated. The reason could be traces of remained Effectene®-mix which affected the cell adhesion. It was therefore decided to apply the LD standard protocol in further experiments.

The possibility to spot transfection mix on top of dried sample-spots was evaluated. When using the manual approach with standard 2µl tips this procedure functions well, but it was very difficult to touch exactly the same area. It is cheaper because less transfection reagent is needed, but it is much more time consuming and accident-prone.

As mentioned before, samples prepared by LD-method gave much better fluorescent signals than samples prepared by the gelatine method. In this procedure treatment with transfection reagent directly before adding the cells was not necessary, but nevertheless the total amount of transfection reagent and the proportions of EC-buffer, Enhancer and Effectene® in the sample had to be optimised, too. Best fluorescent signals were detected with a final volume of 20% EC-buffer, 3% Enhancer and 14% Effectene® in the sample volume (Figure 19). The amount of Effectene® turned out to be most crucial for the signal quality, but also amount of buffer and Enhancer had to be adjusted. Likewise, the sucrose in EC-buffer, necessary to preserve the LD-samples, was tested out in different quantities. It was determined that a range of 0.2 mol to 0.4 mol sucrose in buffer did not significantly influence transfection efficiency.

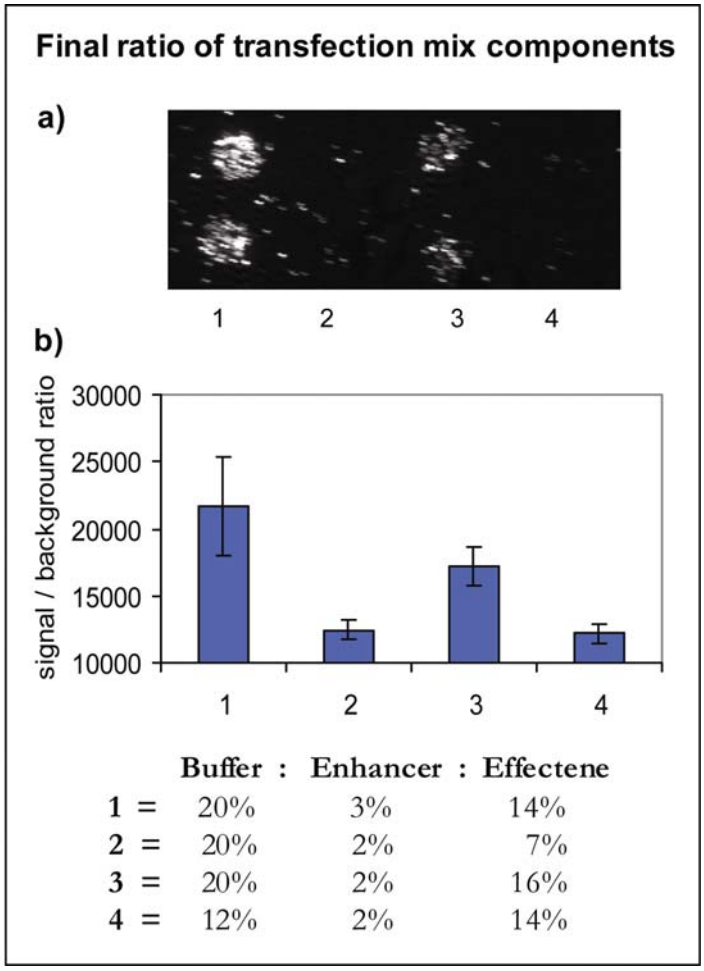

Figure 19

Different ratios of transfection mix components. Figure 19a shows a BIOccd image of the transfected slide, Figure 19b shows the corresponding graph of signal to background ratio of EGFP fluorescence. Optimal results were found with 20% EC-Buffer, 3% Enhancer solution and 14% Effectene®. Lower amounts of Effectene® caused decrease in fluorescence intensity of the spots. The same applies for a lower proportion of the buffer.

3.1.8 Reverse transfection procedure

In the protocol given by Ziauddin and Sabatini (Ziauddin and Sabatini, 2001) 1 x 10^7 cells have to be seeded out one day before reverse transfection. For CAPPIA experiments 1 x 10^7 cells seeded out one day before were compared with those seeded out in a lower density (5 x 10^6 cells) and also with cells taken directly from cell culture on the day of transfection. In the last case especially the monolayer of the cells was of poor quality, whereas fluorescent signals were of good quality. Transfection of the pre-seeded cells at a low density gave an acceptable monolayer, but the fluorescent results of the cells pre-seeded at a high density were better. In this context it made no difference if 1 x 10^7 HEK 293T cells were seeded out one day before or 5 x 10^6 cells were seeded out two days before transfection.

Lengths of transfection incubation from 24 to 96 hrs were tested after placement of the cells on top of spotted slides. When using HEK 293T, the fluorescent signals were still very weak 24 hrs after seeding. Incubation for 4 days resulted in the death of many cells and thus in destroying the monolayer on the slide. This effect could be prevented if media was changed every day. Nevertheless, best results for reverse transfection experiments with HEK 293T were found with time period of 65 hrs and media exchange 48 hrs after transfection.

It turned out that treatment and the number of cells before transfection and the length of incubation were the only parameters which had to be optimised for different cell lines (see section 3.1.9).

3.1.9 Fixation and staining procedures

Fixation

Fixation for fluorescence microscopy is usually done by cross-linking, where special reagents penetrate into the cells or tissues and form cross-links between intracellular components. Reagents are mostly aldehydes, which form covalent bonds between neighbouring amine-containing groups. Beside formaldehyde, standard fixing solutions often contain glutaraldehyde. This is known to be more efficient in preserving cellular structure, but sometimes causes a high background because of unreacted aldehyde groups.

In this study different solutions were tested to fix reverse transfected cells on cell arrays. Fixing solution with addition of glutaraldehyde caused a high fluorescent background compared to fixing with only formaldehyde in the fixing solution (Figure 20). Another tested protocol involved methanol and a cooling step at -20°C. This was very time-intensive, and the fluorescence signals were not better than without methanol and cooling. As expected, the lowest fluorescence signal was detected without any fixing step after transfection.

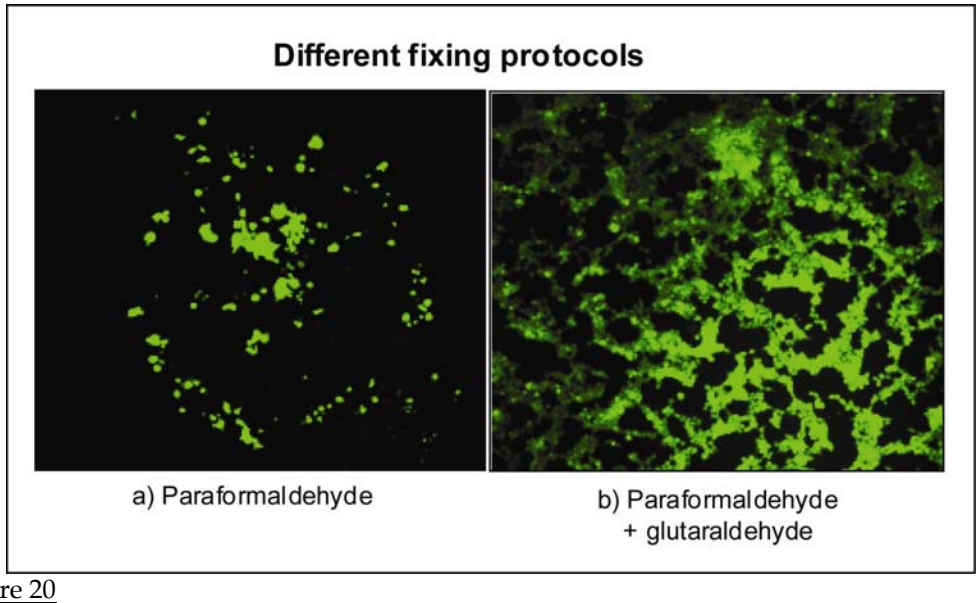

Figure 20

Different protocols to fix cells after reverse transfection. Slides fixed with only paraformaldeyhde in the fixing solution (Figure 20a) showed clearly lower background fluorescence than slides with additional glutaraldehyde in the fixing solution (Figure 20b). Spotted sample was pcDNA4-EGFP. Image of one spot each acquired using microscope.

After fixing, slides were covered with a coverslip, either directly or after immunostaining or staining with DAPI. Slides without coverslip on top (dried cells) gave worse fluorescent signals (Figure 21), while the different mounting media, –such as Fluoromount-G™ (SouthernBiotechnology Associoated, Inc.) and ProLong® Gold (Invitrogen™) – showed no significant differences in terms of the signal intensity. Using PBS as mounting solution resulted in lower intensity of the fluorescence signal.

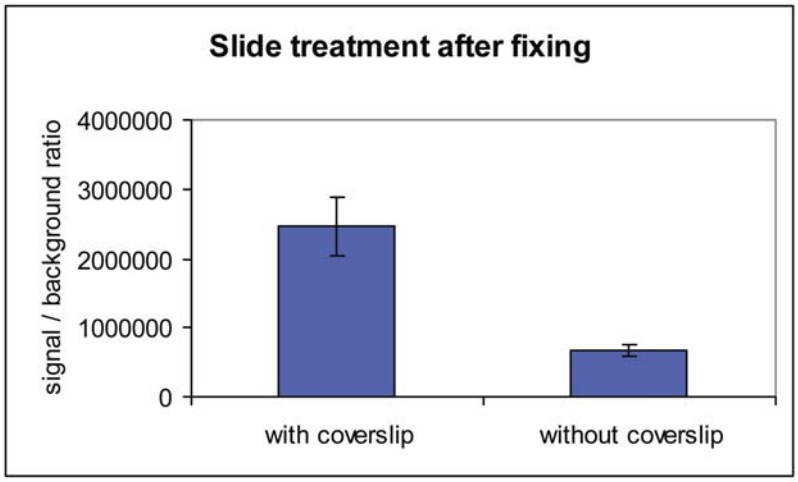

Figure 21
Different treatment of the slides after cells fixing. The intensity of the EGFP signals on slides with a coverslip was much higher than for slides without coverslip.

Immunostaining procedure

When non-autofluorescent plasmids were used for spotting, immunostaining with suitable antibodies was necessary to get fluorescent readout. Different concentrations of antibodies against the expressed proteins were tested to find optimum conditions for immunostaining or alternative detection method (see sections 2.6.2 and 2.6.3). To make the staining procedure as gentle as possible for the sensitive cell monolayer, slides were transferred to another container for solution exchange instead of adding fresh solution in the same container, which had often resulted in cells detaching from the surface.

At the beginning of the project, the *E. coli* ß-D-galactosidase gene lacZ was chosen as a common reporter gene for detecting gene expression for CAPPIA. The best results for immunostaining the lacZ-protein were obtained with mouse anti-lacZ (best concentration 1:250) as first antibody and anti-mouse

Cy™3-conjugated (best concentration 1:800) as secondary antibody. Fluorescence signal of Cy™3 could then be analysed by fluorescence microscopy or BIOccd camera.

A third antibody step with anti-goat Alexa Fluor 488 and also with anti-goat Alexa Fluor 594 (optimum titer at 1:500, respectively) was tested as well as the evaluation of colorimetric detections for CAPPIA detection. Thus, mouse anti-lacZ was used as first antibody and anti-mouse conjugated with alkaline phosphatase as a second antibody (with the titer of 1:50), followed by addition of NBT/BCIP substrate. When reacted with alkaline phosphatase, NBT (Nitro-Blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) together yield a black-purple precipitate, detectable by normal light microscope. The sensitivity of these methods was tested by spotting samples prepared by gelatine method in concentrations of 100 / 70 / 50 / 35 / 25 / 12.5 and 6.25 ng/µl (Figure 22). With a Cy™3-conjugated secondary antibody signals of the precipitate had a distinct intensity down to a concentration of 25 ng/µl and were still detectable at 12.5 ng/µl. At 6.25 ng/µl no signal could be detected. With the additional use of anti-goat Alexa (1:500) as third antibody, the sensitivity was not further amplified and remained in the same range as with two antibodies. Using the colorimetric detection, signals were clearly detectable down to 25 ng/µl. At 12.5 ng/µl and 6.25 ng/µl the signal could not be detected any more (Figure 22). Because colorimetric detection was not more sensitive than immunodetection, this method using non-autofluorescent reporter was chosen for CAPPIA experiments.

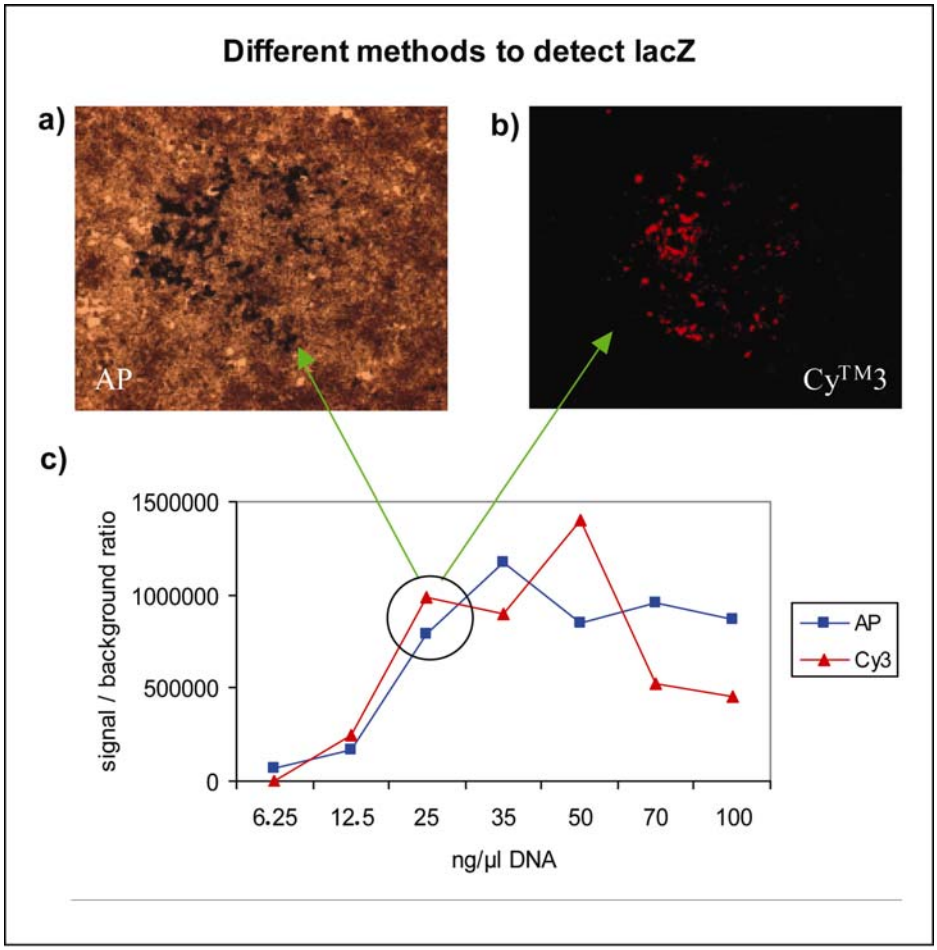

Figure 22

Different methods to detect signal of non-autofluorescent lacZ. PGAL/lacZ samples were spotted in concentrations from 6.25 to 100 ng/µl in order to compare immunostaining using a CyTM3-conjugated second antibody (CyTM3) and colorimetric method using alkaline phosphate conjugated secondary antibody and substances NBT/BCIP (AP). The colorimetric method was not more sensitive than immunostaining and was advantageous only with higher concentrated samples (70 ng/µl and more). The image of lacZ-signal detected with the AP-conjugated antibody was acquired using a light microscope (Figure 22a). Image of the lacZ-signal detected with the CyTM3-conjugated antibody was acquired using fluorescence microscopy (Figure 22b). Both images show a spot with a final DNA concentration of 25 ng/µl. Figure 22c: Corresponding graph.

Enzymatic Reaction

Enzymatic reaction is an alternative way to detect lacZ expression. Usually, the absorption of blue colour of a cleavage product of X-gal (5-bromo-4-chloro-3-indolyl galctopyranoside) as the result of ß-galactosidase expression caused by lacZ transfection is measured. However, this product is non-fluorescent. The "ImaGene Green™ C_{12}FDGlacZ Gene Expression Kit" from Molecular Probes (Invitrogen™) contains a variant of fluorescein di-ß-D-galactopyranoside (C_{12}FDG), a fluorescent ß-galactosidase alternative for X-gal. Excitation maximum of the product is at 571 nm, emission maximum is at 585 nm. For detection with the ImaGene Green™ kit, the substrate solution has to be added before fixing the cells. Different times of incubation from 5 min up to 2 hrs in a wettish container in cell culture incubator were tested as well as different concentrations (17, 33 and 50 µM). A chloroquine solution can be added in a pre-incubation phase before incubation with the substrate (30 min., 300 µM). This manipulation did not however improve the results.

Enzymatic detection of cell arrays turned out to be very error-prone. Fixing the cells was only possible after substrate incubation. This was problematic because after transfection process and cell grow at a high density the cells tended to detach, especially during liquid exchange and microarray displacement. Beside this, it was very difficult to get any signal at all. After a lot of tests with various parameters (including media without phenol red, without FCS and also media without antibiotics) fluorescent spots could only be seen under the microscope if substrate incubation was not longer than 10 min followed by no fixation or rapid fixation for 5 min. Fixing solution had to be without sucrose and slides had to be evaluated as soon as possible under the fluorescence microscope. But even these signals were not stable and disappeared after one day. To find the reason for this, ImaGene Green™ was used to detect the lacZ signal in direct transfection (see section 2.7). This showed that fluorescence was not stable in the cells but rather "walked" outside. Since on cell arrays only a few cells produce the fluorescent signal, in contrast to direct transfection where most of the cell are transfected, signal intensity was too low and it disappeared after a few hours. Thus the enzymatic reaction detection was excluded from CAPPIA experiments.

3.1.10 Transfection of different cell types

As shown in Figure 24 the reverse transfection protocol for the simultaneous transfection of 3 plasmids (reporter + bait + prey) was optimised in 7 phenotypically different cell lines (HeLa, HEK 293 / HEK 293T, PC-3, WI-38, HepG2, COS 7, and Hekl). Reporter GAL4-pZsGreen was co-transfected with positive control plasmids pBD-p53 + pAD-SV40T or with the negative control plasmids pBD-p53 + pAD-TRAF. Cells that expressed the interacting p53 and SV40T hybrid proteins also expressed high levels of ZsGreen reporter, whereas those cells that were transfected with the plasmids encoding for two non-interacting proteins p53 and TRAF or were growing on non-printed areas of the slide, exhibited background fluorescence.

Interestingly, sample and slide preparation were the same for all tested cell lines tested and only the number of cells and time of transfection had to be optimised for each cell type. This is of particular interest for interactions that are dependent on cell-specific post-translational modifications of expressed proteins or that depend on cell-specific co-factors. Cell type dependent differences in PPI-induced reporter expression are already evident for p53 and SV40T proteins that induced higher reporter levels when expressed in PC-3 as compared to HEK 293T cells, even though both cell lines can be transfected with similar efficiencies, as reflected by the level of NF-κB-induced reporter expression in these cell lines.

In general, PC-3 and HEK 293T were found to give best results. Erfle et al. also observed in their siRNA cell array experiments that HEK cells have higher transfection efficiencies than HeLa and COS 7, the HEK cells however tend to grow on top of each other and adhere less than HeLa cells (Erfle et al., 2004). For CAPPIA experiments, HEK was chosen as the standard cell line because of the high transfection efficiency. It has to be emphasized however that overgrowing should be avoided and passages of more than 20 should not be performed. Reverse transfection of HeLa was more difficult to perform because of mostly weaker fluorescence signals than with HEK. COS 7 is a fast growing cell line and thus it was difficult to get a continuous cell monolayer on the slides without the risk of losing cells after more than 48 hrs due to cell overgrowth.

A reverse transfection of fibroblasts (WI-38 and Hekl) could also be performed using the established protocol. WI-38 cells showed clear fluorescent signals for transfection with positive controls pBD-NFkB or pBD-p53 + pAD-SV40T, respectively. It was in similar range as for HeLa and HEK 293, which was sufficient for CAPPIA experiments (Figure 24). Hekl was successfully reverse transfected as well (Figure 23). Fluorescence resulting from reporter expression due to transfection with positive controls was weak compared to the other cell lines, but the proof-of principle was clearly delivered.

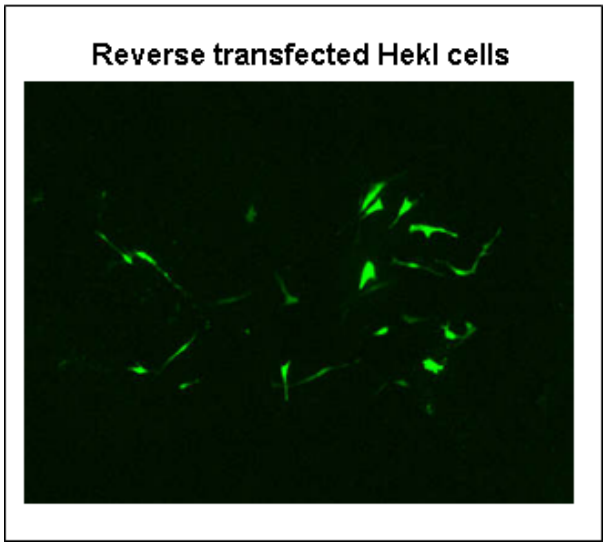

Figure 23
Reverse transfection of fibroblast cell line Hekl. The spotted sample contained pBD-NF-κB plasmid. In co-transfection with reporter plasmid GAL4-pZsGreen, cells expressed fluorescent protein ZsGreen. Image was acquired using microscope.

3.1.11 Transfection of stable transfected cell lines

When co-transfecting three plasmids at the same time the amount of each plasmid has to be lower than in single transfections because of the risk of cytotoxicity. This in turn reduces transfection efficiency. One way to overcome this problem is to stably transfect one of the plasmids into the target cell line.

Firstly, the reporter pGAL/lacZ was used to design HEK 293-LZ cell line. Additionally bait plasmid was stably transfected in HEK 293, creating HEK 293-SMRT and HEK 293-LBD cell lines. All stable transfected cell lines were successfully used in CAPPIA experiments. Fluorescent signals were lower than with HEK 293T as expected, but still well-detectable (Figure 24).

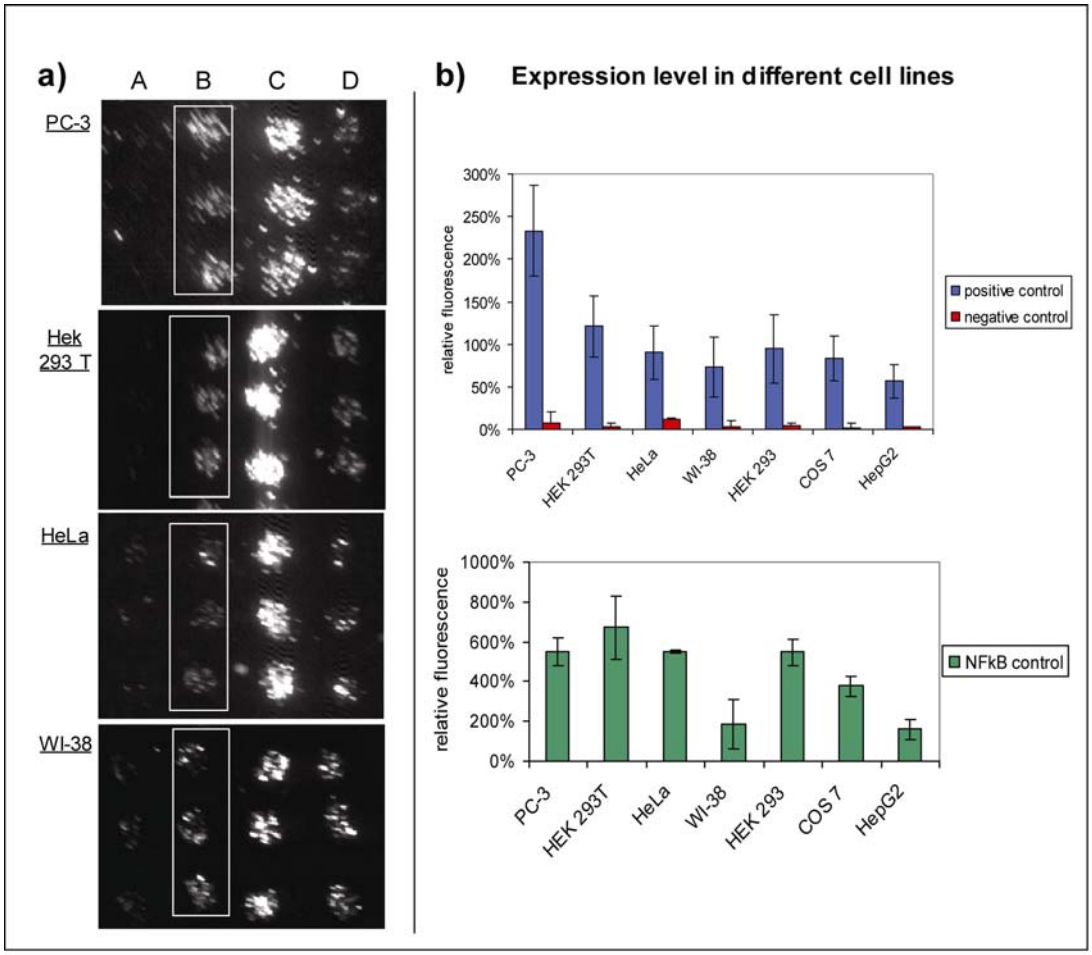

Figure 24

Cell array based PPI screens in different cell lines. Transfection efficiency and specific protein-protein interaction in different cell lines was demonstrated with solutions containing GAL4-pZsGreen reporter and plasmids coding for the known interacting p53 (pBD-p53) and SV40-T (pAD-SV40T) hybrid proteins (boxed, line B). As negative control GAL4-pZsGreen reporter was co-transfected together with the plasmids encoding the non-interacting proteins p53 (pBD-p53) and TRAF (pAD-TRAF) (line A). The pBD-NF-κB control plasmid was used as a positive control to monitor transfection efficiency and reporter performance (line C). EGFP expressing construct was printed as autofluorescent control (line D). Figure a) shows different adherent cell lines transfected using identical microarray slides. Figure b): transfection efficiency differed depending on the cell line tested and was typically lowest for WI-38, COS 7 and HepG2. The highest levels of transfection were repeatedly obtained with the HEK 293T variant that expresses the SV40 large T-antigen. Comparable results were obtained for PC-3, HEK 293 and HeLa cells.

3.2 Screening the prey library for detection of protein-protein-interactions

3.2.1 Verification of known interactions

Next step of developing CAPPIA after optimisation of reverse transfected cell arrays was to explore whether this system allows the verification of known PPI using a small library of cDNA molecules (see section 2.4.3, Table 3). The ligand binding domain (LBD), one of the major domains of the human androgen receptor (AR), was taken as bait and tested against the 17 preys of the library. For that purpose so-called PR-B (prey + reporter + bait) arrays were printed on which all spots contained the same reporter (GAL4-pZsGreen) and the same bait (AR-LBD), but on which each individual spot contained only one of the prey constructs of the library (prey A, B, C etc.). Triplicate spots of each PRB combination, positive and negative controls were printed and used to reverse transfect HEK 293T cells in the presence of 10^{-8} M R1881, a synthetic androgenic ligand.

A strong, androgen-dependent interaction between AR-LBD and AR-NTD (N-terminal domain of AR) could be observed, while no interaction was observed between AR-LBD and the other preys (Figure 25) These findings are consistent with previous published androgen-dependent intra-molecular interactions between the AR-LBD and AR-NTD domains partners (Ahrens-Fath et al., 2005; Doesburg et al., 1997; Langley et al., 1995). The absence of interaction between AR-LBD and the other preys in CAPPIA, either in the presence or absence of R1881 was also confirmed by normal transfections of PC-3 cells using the luciferase reporter plasmid pFR-Luc (Haendler, personal communication).

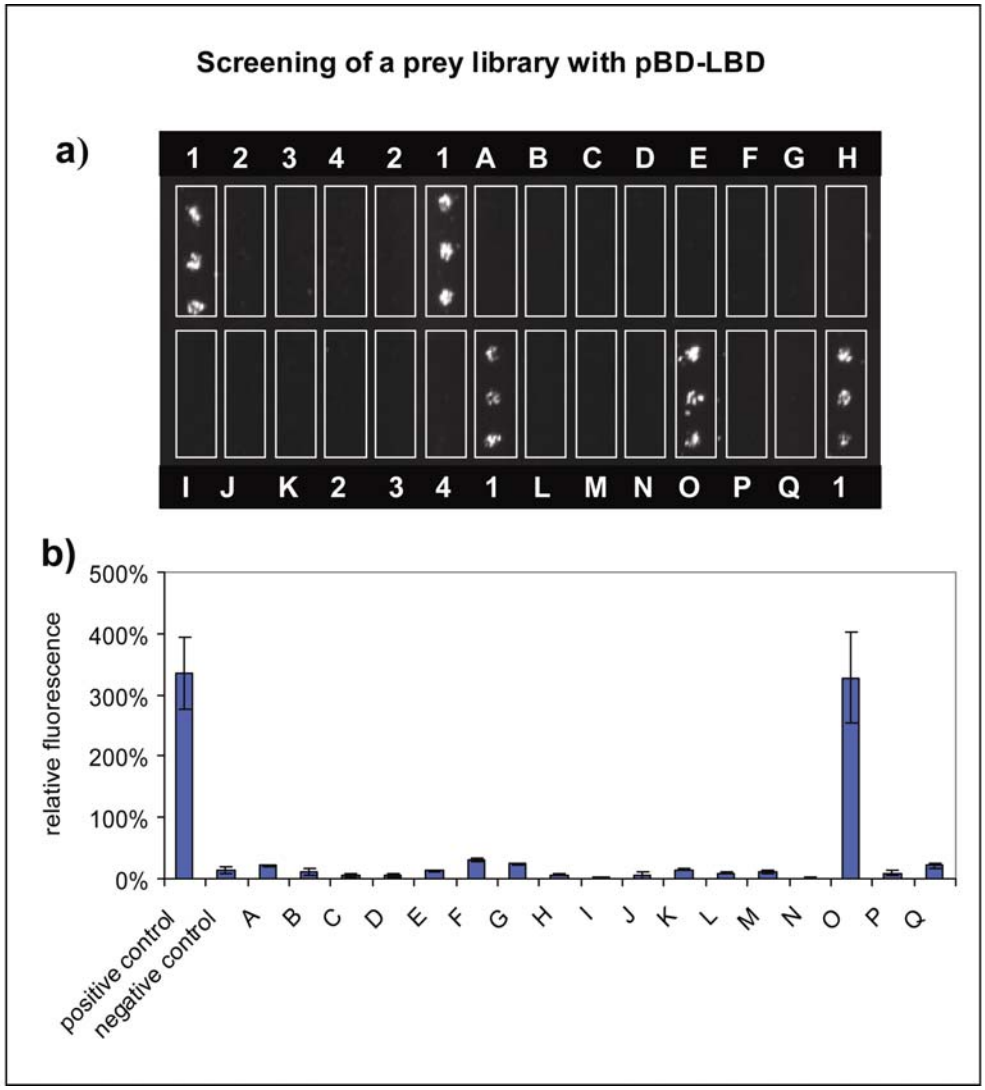

Figure 25

Application of CAPPIA for the detection of hormone-dependent interactions. The AR-LBD bait was co-transfected separately with 17 different preys potentially associated with nuclear receptor function (samples A-Q, see section 2.4.3, Table 3). Triplicate spots of each prey-reporter-bait (PRB) combination (samples A-Q), positive control (1: p53+SV40T) and negative controls (2: p53+TRAF), 3: SV40T and 4: TRAF) were printed and used to reverse transfect HEK 293T cells in the presence of 10^{-8} M R1881 for 3 days. In the presence of the androgenic compound, AR-LBD was found to specifically interact with AR-NTD, the N-terminal domain of the AR. Figure 25a shows BIOCCD scanner image of a representative slide. Figure 25b shows corresponding graph with relative fluorescence signal obtained for the different bait-prey combinations.

3.2.2 Application of CAPPIA for the quantitative detection of hormone-dependent interactions

In order to obtain additional evidence for the physiological significance of the AR-LBD and AR-NTD interaction using cell arrays, a dose-response curve was determined. There GAL4-pZsGreen reporter expression was inducted in a dose-dependent manner in presence of the synthetic agonist R1881 with a maximum response from 10^{-8}M onwards (Figure 26). This was in accordance with previous assays involving normal transfection of both domains and detection of GAL4-induced luciferase activity (Doesburg et al., 1997). Moreover, the dose-dependent inhibitory effects of two antagonists (MPA and OH-Flu) could be reiterated on cell arrays on R1881-induced AR-LBD and AR-NTD interaction (Figure 26). Importantly, the resolution of CAPPIA allowed the detection of quantitative differences in antagonist activity as is reflected by the observation that MPA reached minimum reporter expression at a concentration which is about 2 orders of magnitude lower than OH-Flu as inhibitor. This agrees well with references in the literature that MPA is about 70 times more potent as an inhibitor than OH-Flu, observed after normal transient transfection of both AR domains in CHO cells (Kemppainen et al., 1999).

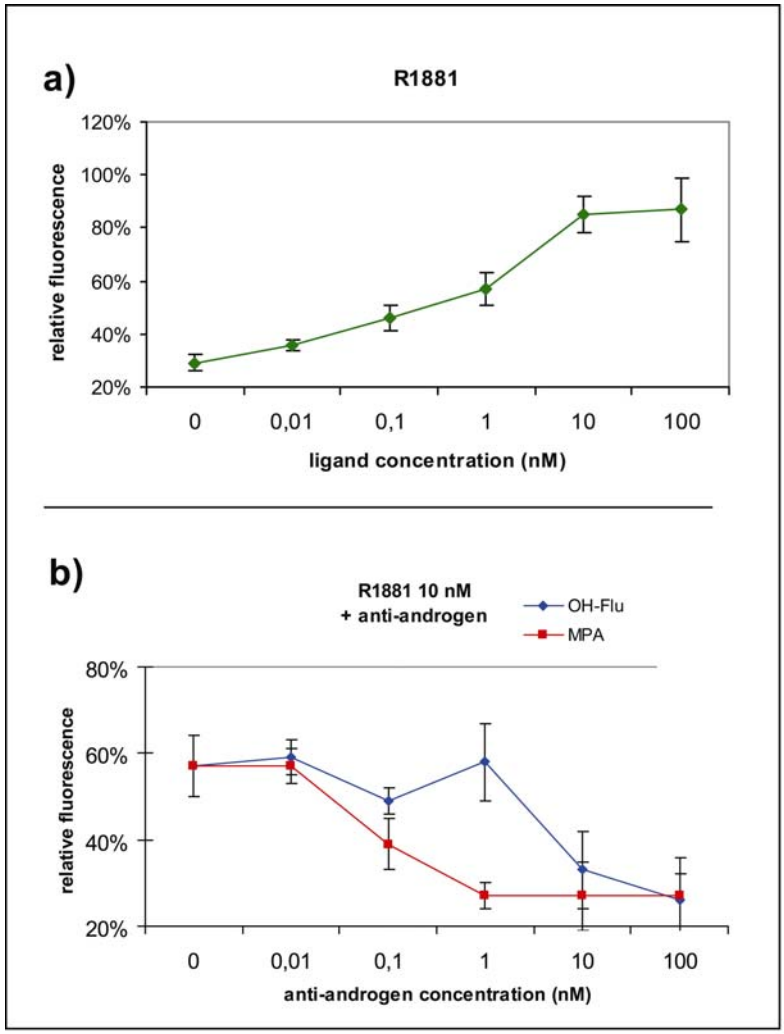

Figure 26

Dose response of AR-LBD and AR-NTD interactions to androgenic and anti-androgenic compounds. AR-LBD and AR-NTD interaction was analysed on cell arrays in the presence of different concentrations of agonist and antagonists. Figure 26a) shows dose-dependent induction of AR-LBD and AR-NTD interaction by the synthetic agonist R1881, showing a maximal response from 10^{-8}M onwards. Figure 26b: Dose-dependent inhibition of the R1881-induced AR-LBD and AR-NTD interaction by two antagonists, medroxyprogesterone acetate (MPA) and hydroxyflutamide (OH-Flu). Quantitative analysis of this inhibition reflects the stronger antagonistic potency of MPA as compared to OH-Flu.

3.2.3 PR-stable-bait and PR-trans-bait cell arrays

In order to increase the possible combinatorial screens for protein interactions using cell arrays and hence further improve the high-throughput application of CAPPIA, alternative slides were printed on which the bait was omitted. Each spot on these so-called prey-reporter- (PR-) slides only contains the reporter and one of the prey constructs. To screen for interacting partners the bait was then introduced into the cells before adding them to the prey-reporter-arrays. This was done by generation of stably transfected cell lines (PR-stable-bait assay). Alternatively, the cells can be transfected transiently with the bait shortly before being added to the arrays (PR-trans-bait assay). In both cases all the cells on these transfected slides express the bait but only those clusters of cells that grow on top of a spot with a prey that can interact with that bait will become fluorescent.

To compare the "PR-stable-bait" and "PR-trans-bait" strategies with the results obtained with the original prey-reporter-bait slides (PRB slides) described earlier (see section 3.2.1), the hormone-dependent AR-LBD and AR-NTD interaction was used. For that purpose, an HEK 293 cell line with a stable integration of the pBD-LBD plasmid was generated (see section 3.1.10) and grown on top of the PR slides. For the PR-trans bait experiment, suspensions of HEK 293T cells were incubated with pBD-LBD plasmid complexed with transfection reagent 5 minutes before adding them to the PR-slides. Finally PRB slides were incubated with non-treated HEK 293T cells as described before. A schematic representation of the three strategies with the corresponding results is shown in Figure 27. After normalisation of the data to correct for differences in transfection efficiencies, the results show that all three strategies resulted in a comparable trans-activation of GAL4-pZsGreen following AR-LBD and AR-NTD interaction.

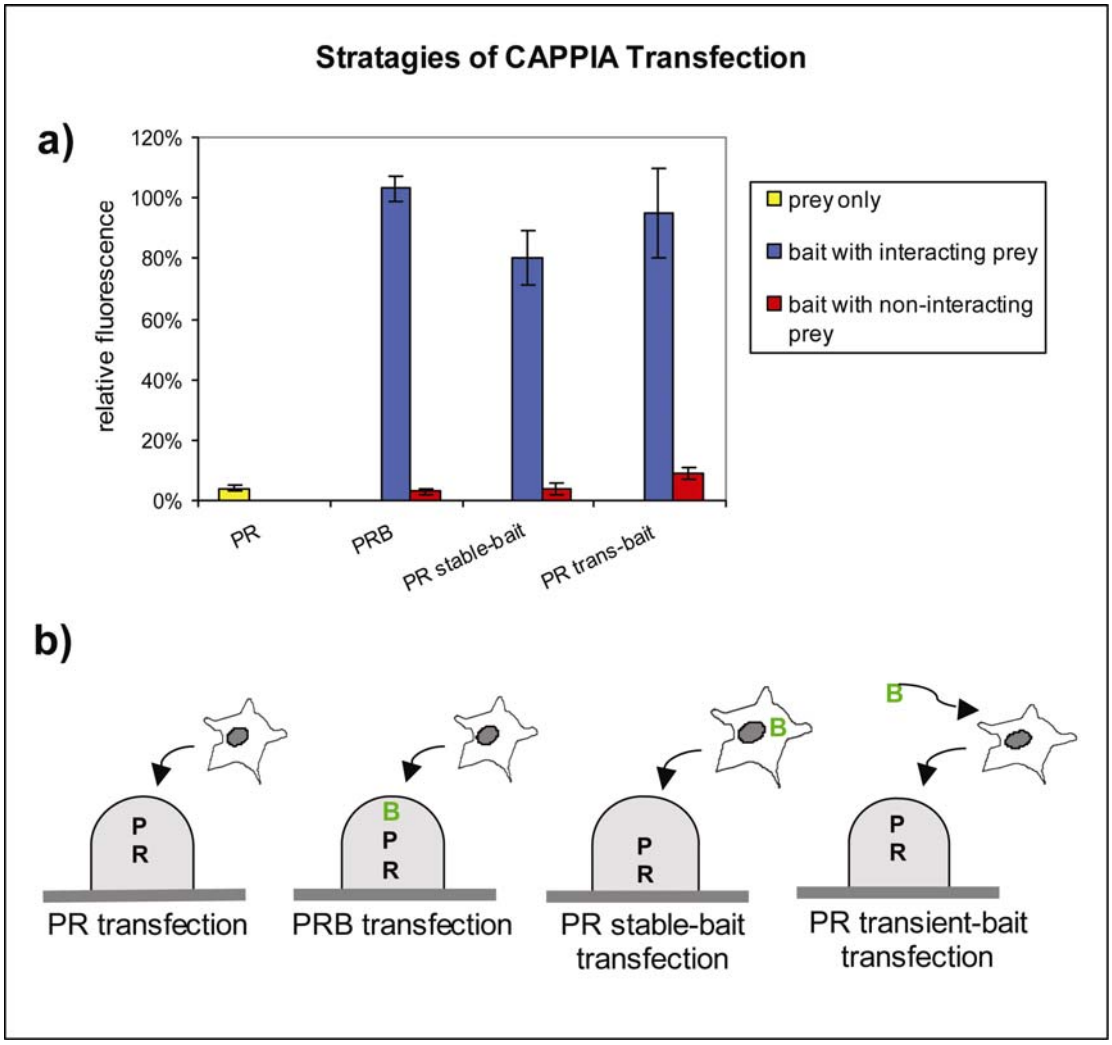

Figure 27

High-throughput screens for PPI using PR-stable and PR-trans bait cell arrays. Different transfection strategies were tested in CAPPIA. On PR-B (Prey-Reporter-Bait) slides bait and prey expression plasmids, AR-LBD and AR-NTD respectively and a reporter plasmid complexed with transfection reagent were immobilized together in array format. In contrast, on PR slides, each spot contains only the reporter and a prey construct. These PR slides were then transfected with cell lines that were either stably transfected with the bait of interest (PR-stable bait) or transiently transfected with it (PR-trans bait). When the data were normalised for differences in transfection efficiencies in the different cell lines, the results showed that all three strategies result in a comparable specific trans-activation of reporter expression following AR-LBD and AR-NTD interaction in the presence of 10^{-8} M R1881. Figure 27a: Graph showing fluorescence signals of reporter protein using different strategies for CAPPIA experiments. Figure 27b: The different CAPPIA transfection strategies.

Chapter 4

DISCUSSION

4.1 Application of VPL slides for reverse transfection

DNA microarrays are an efficient method in molecular biology by which the discovery and analysis of genes entered into a new age. However every microarray experiment is only as good as the material used. Especially the surface on which the DNA is deposited is of high importance. Several groups have worked on increasing transfection efficiency by using different microarray surfaces (How et al., 2004; Isalan et al., 2005; Yamauchi et al., 2004; Yoshikawa et al., 2004). Delehanty and co-workers found that spot size was proportional to substrate hydrophobicity (Delehanty et al., 2004). The largest spots were found with poly-L-lysine, the least hydrophobic compound in test. But they also found that transfection rate was much better with more hydrophobic surfaces. However, depending on the application of the microarray there are different demands for the surface characteristics. For reverse transfection, where cell adhesion is essential, the inventors suggested using GAP slides (Ziauddin and Sabatini, 2001) as alternative to mostly used aminosilane and poly-L-lysine coated slides (DeRisi et al., 1996; Heller et al., 1997; Murphy, 2002; Schena et al., 1995). But GAP slides are relatively expensive, thus increasing the costs of high-throughput experiments like CAPPIA. The self-made VPL slides (surface covered with VECTABOND™ and poly-L-lysine) were at least as good as GAP™ slides from Corning® terms of cell monolayer and transfection efficiency with a better cost/performance ratio.

As shown in section 3.1.9, VPL slides were successfully used for reverse transfection experiments with various cell lines (see also section 4.2). This involved cells, which are standard for transfection experiments like HEK, and also fibroblasts like WI-38, which are generally difficult to transfect. All tested cell lines created good monolayers and expressed fluorescent proteins of the transfected plasmids very well on VPL slides. Similar expression results were obtained for both manual and robotic spotting (either with contact spotting system or non-contact spotting system). Cells remained on the VPL surface after the intensive treatment of immunostaining or other chemical procedures (see section 3.1.8.). Qualities achieved using slides with poly-L-lysine alone

(self-made or commercial) were never as good as with VPL slides. VECTABOND™ is described to enhance adherence of cell preparations to glass slides (Walsh and Wharton, 2005) and thus to improve the qualification of poly-L-lysine slides for cell array experiments, which was confirmed in the study presented here. Treatment of the slide surface with silane did not result in good fluorescence signals, suggesting that cells cannot pick up the spotted DNA efficiently from these surfaces. Moreover, VPL slides can be stored for a long time before or after spotting (more than 3 months in each case) – while still retaining their maximum cell adhesion properties. This makes design of experiments more flexible in terms of preparation of large numbers of samples in advance, thus reducing overall costs. They can then be used for various experiments at any time as needed, or sent all over the world to groups not equipped with automated spotting systems.

Not only slides are used for reverse transfections. For examples Hodges et al. used poly-L-lysine coated slides with 50-well silicon gaskets on top in their protocol (Hodges et al., 2005). But even if this or other modifications seem to be practical to compare media containing different components like for example different hormones, this is a drawback for high-throughput research because of the very high price for every experiment. Furthermore using another format could cause problems with scanning systems as well as it increases the number of cells needed when the format is larger than a slide.

Definitely more suitable for experiments with different components in the media is the Lab-Tek™ Chamber Slide™ system from Nunc™ with separated wells. Even when not VPL-coated, this system gave good results for adhesion of the cells as well as for fluorescence signals in reverse transfection experiments with the advantage of having standard slide format. However, as mentioned before, best results for all parameters were found when the slide surface was coated with VPL. As containers, the Quadriperm boxes offer advantages over Square dish boxes. In a Square dish box 1×10^7 cells have to be added even for only one slide. For comparing different components in the media, this number of cells is necessary for every single approach. In contrast, in a Quadriperm container only 3.5×10^6 cells per slide are necessary. It has enough space for 4 different cell arrays at the same time to compare different parameters in parallel.

Summarising, the combination of VECTABOND™ and poly-L-lysine (= VPL) forms the best slide surface for reverse transfection. It can be used for standard as well as for cell types, which are difficult to transfect, for example fibroblasts. Being cost-effective, easy to make and suitable for long term storage, VPL slides meet all requirements for high-throughput research.

4.2 Reverse transfection of different cell types

Recently, the importance of using *"a wider range of cell types"* in cell-based microarrays was pointed out (Palmer and Freeman, 2005). Currently, most of the experiments are performed with HEK cells only, which is certainly a limitation of these assays. The group of Sabatini overcame this problem by using lentiviruses instead of conventional transfection, and was able to transfect primary cells efficiently (Bailey et al., 2006).

Another microarray-based transfection method called the surface transfection and expression protocol (STEP), is comparable to the system of Ziauddin and Sabatini, showed to be very efficient for neuronal cell lines (Redmond et al., 2004). Likewise, the reverse transfection protocol presented in this thesis is suitable for transfecting different types of cells. This has been demonstrated in section 3.1.9 for HEK 293T, HEK 293, HeLa, HepG2, PC-3, COS 7 and also fibroblasts (WI-38 and Hekl). Sample and slide preparation procedure were the same for all tested cells, only the number of cells and time of transfection had to be optimised for each type. All of the cells were well-adherent to VPL slides, the transfection was efficient and fluorescent results were satisfactory and reproducible. Therefore it might be assumed that the protocol presented in this study may also be suitable for other adherents.

Alternatives to chemical transfection applied here (see section 3.1.6) were recently developed to increase transfection efficiency. Chang et al. used a single-step matrix-surface-mediated transfection ("surfection") to transfer multiple plasmids into cells in array format by coating cationic polymers on the surface of substrates (Chang et al., 2004). Another innovation, called magnet-assisted transfection (MaTra, IBA GmbH), makes use of magnetism to transfect cells (Liman et al., 2005). With some modifications this could be applied in CAPPIA as a future development. This is maybe of particular interest for cell lines, which are difficult to transfect, when chemical-driven transfection does not work.

Currently, the reverse transfection is limited to adherent cells. To overcome this limit, the study of Kato et al. seems to be a promising approach (Kato et al., 2003). The authors presented coverage of the glass surface with BAM (biocompatible anchor for membrane) for attachment of non-adherent cells. If this could also be optimised for the CAPPIA protocol, potentially every cell line could be used for reverse transfection. But even now the potential of CAPPIA is enormous. The fact that specific protein-protein-interactions can readily be analysed in so many different cell lines without modification of the printed slides as shown before is of particular interest for interactions which depend on cell specific post-translational modifications of the expressed proteins or on cell specific co-factors or co-activators.

4.3 Reporter expression induced by bait and prey interaction

4.3.1 Multiplex plasmid transfection

One of the main challenges in establishing CAPPIA was the co-transfection of 3 plasmids (bait, prey and reporter) at the same time. It turned out that the ratio of the plasmids is important as well as the total amount of DNA. As shown in section 3.1.3 a final concentration over a certain level can cause toxicity. But when the amounts of each of the 3 plasmids are low, concentrations of expressed bait and prey are not high enough to activate sufficient reporter expression and finally to obtain a good fluorescent signal. One solution was to do CAPPIA experiments with spotting only 2 plasmids (prey and reporter) on PR-slides and transfect cells stably or transiently with the bait (see section 4.4.5).

However, co-transfecting 3 plasmids at the same time were successfully done with both the gelatine method and the LD-method, of which LD-method mostly gave stronger signals. Gelatine method was superior to LD-method only when spotting was done with the VersArray ChipWriter Pro robot system (see sections 3.1.3 and 4.3.3). In the other cases (robot spotting with the sciFlexArrayer piezodispenser and manual spotting) the LD-method gave stronger fluorescent signals. Thus, although sample preparation is more expensive with the LD-method than with the gelatine method, it was adopted as standard for CAPPIA. The advantage is also that no additional step between spotting and reverse transfection is necessary since transfection reagent is already in the samples. Storage conditions for the spotted slides (dark, cold, and dry) of both methods remain the same (see section 3.1.5).

4.3.2 Advantage of fluorescent reporter

Initially pGAL/lacZ was used as reporter plasmid. Various methods were compared for sensitivity to detect the expression of the lacZ protein (see section 3.1.8). All lacZ-detection methods (immunofluorescence or enzyme-activity-based) are time consuming and carry the risk of delamination of the cell monolayer during the process of signal development. The lacZ reporter was already described as disadvantageous compared to GFP-based reporters (Hobert and Loria, 2006).

In order to improve cell array methodology and to enhance its robustness, reporter system of CAPPIA was converted to an autofluorescent-based reporter. Two plasmids were designed and tested (see section 3.1.4), of which GAL4-pZsGreen proved to be the most applicable. The fluorescence signals of expressed ZsGreen protein were capable for direct analysis by suitable scanning system or fluorescence microscope. This is of particular advantage in high-throughput systems such as CAPPIA.

4.3.3 Microarray spotting and storage

As described in section 2.5.3 and tested in section 3.1.5 slides were spotted in two ways: With a robot or manually. Undoubtedly manual spotting is not suitable as high-throughput tool, but it turned out to be very helpful in the optimisation phase, especially when only a few samples have to be spotted on a slide, e.g. for tests with new cell lines or different components in the media like different hormones (see sections 3.2.1 and 3.2.2). Advantages of manual spotting are the independence from automated systems and the easy and quick production of small numbers of slides. No special equipment is needed, and the technique can be quickly established as routine. Thus the manual procedure is a supplementary tool for cell array experiments even after the phase of optimising CAPPIA.

Nevertheless, automated slide spotting is indispensable for high-throughput functional genomics. Tested systems for this were contact and non-contact DNA-printing. The contact spotting robot systems for microarrays, the VersArray ChipWriter Pro, created slides of good quality at best with four times repetitive spotting and the use of SMP4B pins (see section 3.1.5). However, this system was only suitable for spotting samples prepared by the gelatine method and failed with LD-samples (signals were always too weak). The non-contact sciFlexArrayer piezodispenser created reliable cell arrays also with LD-samples. It was routinely used for CAPPIA experiments due to its high flexibility.

One main advantage of CAPPIA is that the particular steps of the procedure can be separated in time. This is because of long storage possibility of the slides (over month) before and after spotting (see also section 4.1). Complex or cost intensive material is not necessary; a refrigerator, some aluminium foil and a box of dry pellets are sufficient to keep slides dark, cool and dry. When slides have been stored in this way the fluorescent signals were still very stable after cell fixation and covering the slides with mounting media and coverslip.

4.3.4 Fixation and staining

Different cell fixation procedures were compared. Standard approach involved 20 minutes fixing in 3.7% formaldehyde and 4% sucrose in 1x PBS (see section 3.1.8). Even when fluorescence signals seems to be slightly stronger with methanol fixation, the protocol without methanol in the solution and cooling at -20°C was chosen because it is faster, easy and effectual and thus very convenient for high-throughput experiments. Fixation with additional glutaraldehyde turned out to be worse than the other methods because of a very high background signal.

However, the most sensitive step in cell arrays experiments in this thesis was the immunostaining process with multiple washing and blocking steps in-between. Even when moving the slide carefully from one container to another instead of changing solutions in the same container, this procedure often led to cell monolayer detachment. Thus the conversion to autofluorescent reporter (see section 4.3.2), which makes the whole procedure unnecessary, increases the robustness and simplicity of CAPPIA, and also saves time. If cells do not have to undergo staining after the fixing step, cell array experiments can be done within just 4 days - from preparing of the sample solutions and spotting till analysis with microscope or BIOccd camera, during which transfecting the cells for 3 days takes most of the time. Immunostaining would require extension of the procedure for another day.

4.4 CAPPIA and detection of protein-protein-interactions

Cell arrays allow the simultaneous transfection and subsequent analysis of large numbers of different cDNA constructs into adherent mammalian cells. This makes them suitable for high-throughput analyses of protein-protein-interactions. CAPPIA as the combination of cell arrays and the mammalian two-hybrid system makes use of this feature.

4.4.1 Verification of known interaction using CAPPIA

After optimisation, the CAPPIA method was used to screen a library of 17 cDNA molecules coding for proteins or protein domains potentially associated with nuclear receptor function. The mammalian two-hybrid system has already been described for investigation of human nuclear receptors. For example Petterson et al. focused on dimerisation of the estrogen receptor (Pettersson et al., 1997) and Leonhardt analysed the role of agonists and antagonists for dimerisation of progesterone receptor (Leonhardt et al., 1998).

In the present work one of the major domains of the human androgen receptor, the ligand-binding domain (AR-LBD), was tested as bait against the 17 preys of the library listed in Table 3 (see section 2.4.3). One of these preys was the N-terminal domain of the androgen receptor (AR-NTD). The known ligand-dependent interaction of AR-LBD and AR-NTD (Ahrens-Fath et al., 2005; Doesburg et al., 1997; Langley et al., 1995) was verified (see section 3.2.1). Similar to other two-hybrid systems, transcription of reporter gene occurred only when the transfected chimeric proteins of bait and prey interacted. This and the verification of the absence of interaction between AR-LBD and the other preys in the tested library, either in the presence or absence of R1881 as ligand, shows the reliability of CAPPIA and its suitability for large scale detection of protein-protein-interactions, even if further components are necessary for these.

4.4.2 Determination of essential components for protein-protein-interactions

For PPI detection systems with only on/off signalling can also be used (interaction/no interaction). But CAPPIA seems to be more effective. As shown in section 3.2.2 it detected the interaction of AR-LBD and AR-NTD in the presence of synthetic agonist R1181 in a dose-dependent manner with a maximum response beginning at 10^{-8}M, in accordance with previous studies (Doesburg et al., 1997). The fact that the resolution of CAPPIA allows the detection of quantitative differences also in antagonist activity, as is reflected by the observation that MPA was about 100 times more potent than OH-Flu as an inhibitor, is of great importance to show sensitivity and reliability of CAPPIA. Similar differences in inhibitory potency between MPA and OH-Flu have also been observed after normal transient transfection assays of both protein domains in CHO cells (Kemppainen et al., 1999). Taken together, these experiments clearly demonstrate that cell arrays provide a functional readout to monitor PPI under different physiological conditions. They can be used to screen ligand-dependent PPI and to quantify the dose-response of these interactions to various compounds.

4.4.3 Screening for unknown protein-interaction partners

The results presented in this study strongly suggest that CAPPIA can successfully being used for identification of unknown protein-protein-interactions. Currently, large bait-prey libraries are being generated for use in CAPPIA using the Gateway® (Invitrogen™) compatible destination pCMV-AD and pCMV-BD vectors and panels of pENTRY clones. Since more than 15 000 open reading frame clones are available as sequenced open reading clones in pENTRY vectors (RZPD Berlin, Germany), the Gateway system allows the fast and efficient construction of prey and bait libraries of choice, e.g. representing transcription factors and cell signalling factors.

4.4.4 High-throughput character of CAPPIA

In high-throughput studies not only large amounts of data have to be collected, but also the quality of the data has to achieve a certain standard. Furthermore it is advantageous when the methods used are low-cost, easy to provide and perform, flexible, and fast. CAPPIA was found to meet all these demands. It convinces especially because of its flexibility and the unmatched efficiency in terms of costs per analysed sample, particularly compared to small-scale mammalian two-hybrid systems (Leonhardt et al., 1998; Luo et al., 1997). Past modifications of the mammalian two-hybrid (Fotin-Mleczek et al., 2000; Shioda et al., 2000), even with the aim of larger scale research (Liebel et al., 2003; Suzuki et al., 2001; Zhao et al., 2004), are still limited, not least because of the need for automation of liquid dispensing and a significant consumption of reagents for this microwell plate-based approach (Shioda et al., 2000; Stelzl et al., 2005). CAPPIA does not need automated liquid dispensing and consumes only nanolitre of sample solution per spot. Even an automated spotting system or an expensive fluorescence microscope is not absolutely necessary: Slides can be spotted by another group and sent to the research group, or the transfected and fixed slides can be sent to a collaborating institute with a fluorescence scanning system.

4.4.5 Stable-bait and trans-bait reverse transfections

Co-transfection of three plasmids (bait, prey and reporter) needs a good optimisation and allows only low concentrations of each plasmid (see section 4.3.1). One strategy to overcome this limit is to stably transfect a cell line with one of the constructs, as done in this thesis. Stable transfected plasmid was the reporter (pGAL/lacZ), similar to Shioda et al. with reporter plasmid GFP (Shioda et al., 2000). The cell line HEK 293 was used for this. It is known to have lower transfection efficiency than its variant HEK 293T (see section 2.1.2), but the neomycin resistance in HEK 293T would make the pre-arrangements for selecting the successful stable transfected cells more time-consuming.

Also the bait plasmid pBD-LBD was stably transfected in HEK 293 cells in order to increase the possible combinatorial screens and hence further improve the high-throughput application of CAPPIA. Stable-bait transfected cells were used in so-called PR-stable-bait assays (see section 3.2.3), where the bait was omitted on the slide and each spot only contains the reporter and one of the prey constructs. Alternatively, the cells can be transfected transiently with the bait shortly before being added to the arrays (PR-trans-bait assay). Both cases were successfully performed in this thesis by using the hormone-dependent interaction of AR-LBD and AR-NTD. Comparison of PR-stable-bait and PR-trans-bait strategies with the results obtained with the original prey, reporter and bait plasmid slides (PRB-slides) shows that although PRB slides offer a cost-effective and robust platform for simultaneous comparison of large numbers of interactions in different cell lines and under different culture conditions (time of culture, addition of agonists or antagonists), the PR slides are more suitable for the large-scale screening of novel bait-prey interactions. Up to 900 features can be spotted per slide so that each slide represents comprehensive collections of preys. Since there is no bait on PR-slides these libraries can be screened with any bait of interest, further increasing the high-throughput application of CAPPIA.

In addition PR-slides can be printed in large batches and can be stored for a long time as discussed before, thus making CAPPIA even more cost effective. So far, no other system to detect PPI in mammalian cells has reached the degree of flexibility characteristics of CAPPIA.

4.5 Advantages and limitations of CAPPIA

CAPPIA is a newly developed method for high-throughput analyses of protein-protein-interactions directly in mammalian cells. It was demonstrated that it is a serious alternative to the yeast two-hybrid, where false positives are still a problem, especially because of the vast quantity of data produced. Current high-throughput research in yeast focuses on testing the numerous detected interactions with different methods (Colland et al., 2004; Stelzl et al., 2005). This can in turn mean that many important interactions may be missed in such screens, contributing to the highly false negative rate (see section 1.4.2). Leonhardt et al. described a discrepancy between yeast- and mammalian-based two-hybrid studies relating to nuclear receptors and suggest that it is *"likely due to receptors in yeast possessing lower intrinsic transcriptional activity due to the absence of steroid receptor coactivators that are normally present in mammalian cells"* (Leonhardt et al., 1998).

Instead of trying to improve reliability of the yeast two-hybrid, it seems to be more reasonable to invest more effort in developing mammalian-based systems, which offer the advantage to test mammalian PPI within a native cellular context. CAPPIA addresses this issue, especially because phenotypically different cell types can be used. The same array can be used for revealing cell type specific protein-protein-interactions. Furthermore, the number of false positives can be significantly reduced with CAPPIA by using slides with only prey and reporter plasmids (PR-slides). Interrogated with different baits and different cells, they allow a fast and easy detection of protein-protein-interactions. As such, preys interact with unrelated baits, so-called bait-unspecific false positives (Ito et al., 2001a; Serebriiskii and Golemis, 2001), can be distinguished without need for further evaluation.

Beside the advantages of the mammalian two-hybrid character, CAPPIA has the advantage of using microarrays. Indeed, CAPPIA slides are printed with the same robotic devices used for production of conventional DNA microarrays. Furthermore, cell arrays require far less DNA, transfection reagents and cells as compared to assays performed in microwell plate format. The high number of prey-bait combinations that can be achieved using microarray technology allows slide to be produced with an equivalent capacity of 9 standard 96 microwell plates. The number of preys per slide can further be increased by pooling 3 preys per feature. In conjunction with the idea of

pooling samples on arrays Schmid et al. recently published a technique called "feature multiplexing" (Schmid et al., 2006). In this encoding system the density of probes will be increased by incorporating multiple probes into different features.

But even though cell-based microarrays are a powerful technology with great potential only a few papers have been published since the original study of Sabatini`s group. The reason for this could be the significant requirement for costly hardware like microarrayer, scanner and/or microscope system with image analysis software (Palmer and Freeman, 2005). Hence, only a few laboratories may have the resources to set up this technology. The protocol for CAPPIA experiments used in this thesis overcomes this limitation. For small amounts, manual spotting is a useful alternative to robot produced slides, and for screening on a high-throughput scale it is possible to get pre-made microarrays from a service facility, which can be then stored for several months. Only standard cell culture equipment is then necessary to transfect these slides, in the case of PR-slides with any bait of choice.

The use of an autofluorescent-based reporter in CAPPIA further increases the speed and cost-effectiveness of the assay and reduces material consumption. Indeed, using these slides, protein-protein-interactions can be detected without the need for immunofluorescence staining or enzyme-based reporter detection, and signal detection is performed using common DNA array scanners or high-throughput microscopy. Even this equipment is not absolutly necessary because fixed slides can easily be transported to another laboratory for further analysis. Thus CAPPIA is feasible also for groups with low budget, in contrast to microwell-based systems like the one described by Suzuki et al. (Suzuki et al., 2001). The authors described a PCR-mediated preparation system based on the mammalian two-hybrid method. It allows for the rapid preparation of high numbers of bait and prey samples, but since performed in microwells, it still requires semiautomatic multiple dispensers as well as a lot of reagents for the downstream enzymatic detection of interacting proteins. Capability of CAPPIA is definitely comparable to that of enzyme-based mammalian two hybrid assays performed in microwells, allowing the analysis of quantitative responses of interactions to various compounds. No extensive liquid handling infrastructure is required once the slides have been printed. In addition PR- or PRB-slides can be printed in large batches and stored frozen

without losing their efficiency, increasing the flexibility and cost effectiveness of CAPPIA.

In addition to its advantages, CAPPIA has also some limitations. Similar to other two-hybrid systems this assay is not appropriate for the analysis of fusion proteins, which are not translocated to the nucleus. Furthermore, CAPPIA is not suitable when the transcription factor domain occludes the important site of interaction, a limit of all systems working with the two-hybrid principle.

Taken together, the high capacity of the cell arrays together with the flexibility to interrogate any bait of interest and the small amounts of reagents that are required makes CAPPIA currently the most economical high-throughput detection assay for protein-protein-interactions in mammalian cells.

4.6 Outlook

Because of the high degree of flexibility, the CAPPIA protocol can be used for various applications. One field for CAPPIA experiments is the screening of prey libraries to find protein partners for a specific bait of interest. Also building up or increasing networks of PPI by testing different plasmids against each other might be possible when using this method. As described in section 4.4.3, large bait-prey libraries could be generated in a fast and efficient way by using Gateway® (Invitrogen™) compatible vectors and panels of pENTRY clones.

Another area of application for CAPPIA could be screening for new drug targets or other compounds involved in known PPI. As shown in section 4.2, many different cell lines can be used for this purpose, making this system attractive for clinical research. Knowledge of protein interactions can shed light on diseases that arise when these interactions are disrupted or deregulated, and microarray-based systems like CAPPIA could lead to intensification of studies of functional protein complexes. In addition this method could also offer an economical alternative for the identification of potential drug targets when combined with screens for small molecule ligands that can disrupt or modulate interactions of interest.

The current achievable CAPPIA density of about 900 spots per slide can be increased for example by pooling preys on PR-slides (see section 4.5). This should be one of the next steps in CAPPIA experiments. Furthermore, as already mentioned (see section 4.2), another possibility could be re-evaluation of chemical transfection process in general. The method of magnet-assisted transfection (Liman et al., 2005) seems to be a very efficient form of cell transfection. Future development of chemical transfection should be kept in view to upgrade CAPPIA in due time, likewise the reagent for attachment of non-adherent cells developed by Kato et al. (Kato et al., 2003). It shows another direction of CAPPIA development with application of clinically relevant cells such as lymphocytes.

Summarising, CAPPIA showed successfully its potential as a high-throughput method to detect PPI directly in mammalian cells. Nevertheless, it has to assert itself in future research. But because of its low equipment and material requirements and the high degree of flexibility, CAPPIA could definitely increase the number of investigations based on cell arrays and thus makes a contribution to explore the knowledge of functional genomics.

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APPENDIX

ACKNOWLEDGEMENT

LIST OF FIGURES

LIST OF TABLES

CURRICULUM VITAE

PUBLICATIONS

SELBSTÄNDIGKEITSERKLÄRUNG

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LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

°C	Degree Celsius
µ	Micro
AD	Activation domain
AR	Androgen receptor
B	Bait
BD	Binding domain
Bidest	Bi-distillated water
bp	Base pair
BSA	Bovine serum albumin
C. elegans	*Caenorhabditis elegans*
CAPPIA	Cell array based protein-protein-interaction assay
cDNA	Complementary DNA
COS 7	African green monkey kidney cell line
Cy3	Fluorescent cyanine dye 3
D. melanogaster	*Drosophila melanogaster*
DAPI	4`,6-diamidino-2`-phenylindole dihydrochlorid
DBD	DNA binding domain
DHT	5α-dihydrosterone
D-MEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DsRed	Red fluorescent protein from *Discosoma* sp. reef coral
E. coli	*Escherichia coli*
EGFP	Enhanced green fluorescence protein
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
GAP slide	Gamma amino propyl silane coated slide
GR	Glucocorticoid receptor
HcRed	Red fluorescent protein from *Heteractis Crispa* reef coral
HEK	Human embryonic kidney
Hekl	Human skin fibroblast
HeLa	Human epithelial cells, fatal cervical carcinoma

HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
HepG2	Human hepatocellular carcinoma
HPRD	Human Protein References Database
HSP	Heat-shock protein
IκB	Inhibitor -kappaB
lacZ	ß-D-galactosidase gene
LBD	Ligand-binding domain
LD	Lipide DNA
Luc	Luciferase
M	Moles per liter
mM	Milimoles per liter
MPA	Medroxyprogesterone acetate
MPIMG	Max Planck Institute for Molecular Genetics
n	nano
NCoR	Nuclear hormone receptor corepressors
NF-κβ	Nuclear factor -kappaB
NTD	N-terminal activation domain
OH-Flu	2-hydroxyflutamid
P	Prey
PBS	Phosphate buffered saline
PC-3	Human prostate epithel
PCR	Polymerase chain reaction
PEI	Polyethylenimine
pLacZ	pcDNA3.1D/V5-His/lacZ
PPI	Protein-protein-interaction
PRB-slide	Prey + Reporter + Bait spotted slide
PR-slide	Prey + Reporter spotted slide
R	Reporter
R1881	Methyltrienolone
RLuc	Renilla luciferase
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Revolutions per minute
RRS	Ras Recruitment System
RZPD	Deutsches Ressourcenzentrum für Genomforschung = German Resource Center for Genome Research

SH3 domain	Src homology 3 domain
siRNA	Small interfering RNA or short interfering RNA
SMRT	Silencing mediator of retinoid and thyroid receptor
SNP	Single nucleotide polymorphism
SRS	SOS Recruitment System
STEP	Surface transfection and expression protocol
SV40T	Simian virus 40 large T antigen
TAD	Transactivation domain
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
VP16	Etoposide phosphate
VPL slide	VECTABOND™ Reagent + Poly-L-Lysine coated slide
WI-38	Human lung fibroblast

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PUBLICATIONS

Articles

A. Fiebitz, L. Nyarsik, B. Haendler, Y.H. Hu, F. Wagner, S. Thamm, H. Lehrach, M. Janitz, D. Vanhecke (2006): High-throughput mammalian two-hybrid screening for protein-protein interactions using transfected cell arrays
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Berlin, 6. – 9.9.2006
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GBM Annual Fall Meeting

Berlin, 4. - 7.4.2004
Human Genome Meeting

Paris, 15. - 19.5.2003
FOCIS 3rd Annual Meeting

Berlin, 17. - 19.11.2002
Symposium of the NGFN and DHGP

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Hiermit erkläre ich, dass ich die vorliegende Arbeit eigenständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde.

Berlin, den

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(Andrea Fiebitz)