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.