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.

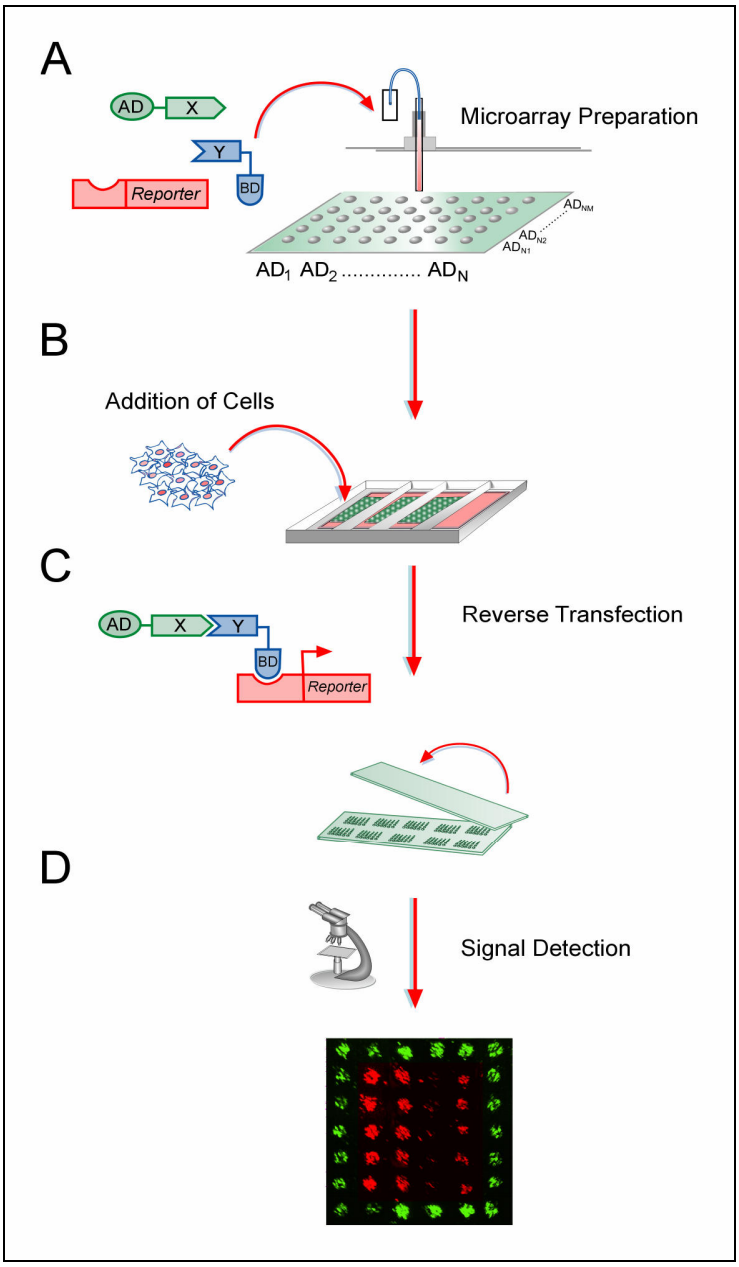

Figure 4

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.

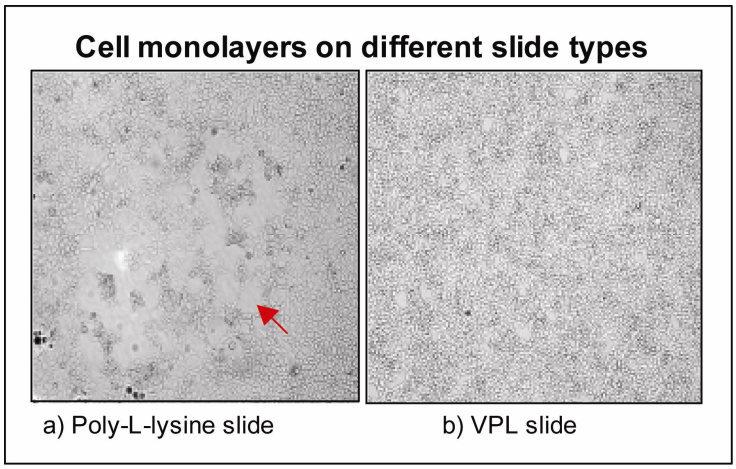

Figure 5

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.

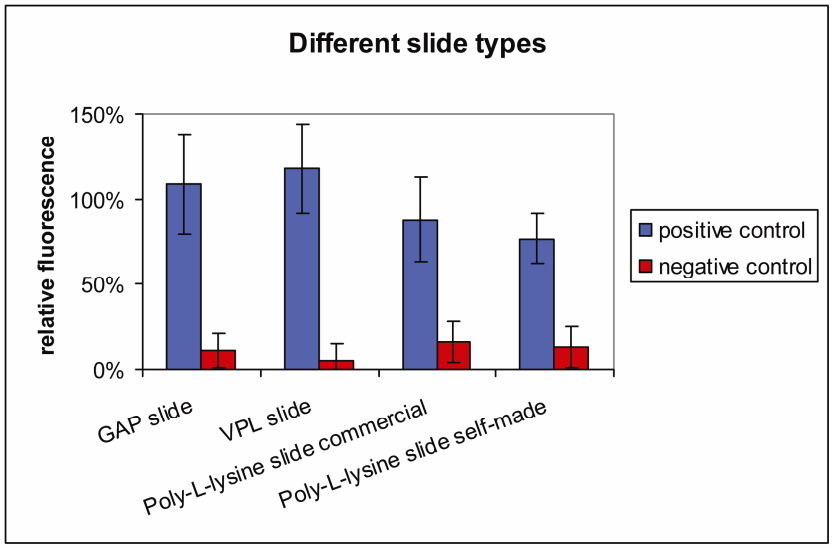

Figure 6
Fluorescent signals from detection of expressed proteins on different slide types. Graph of relative fluorescence of positive and negative controls on GAP™ slides, VPL slides and two types of poly-L-lysine slides (commercial and self-made). Spotted plasmids were pBD-p53 + pAD-SV40T as positive 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).

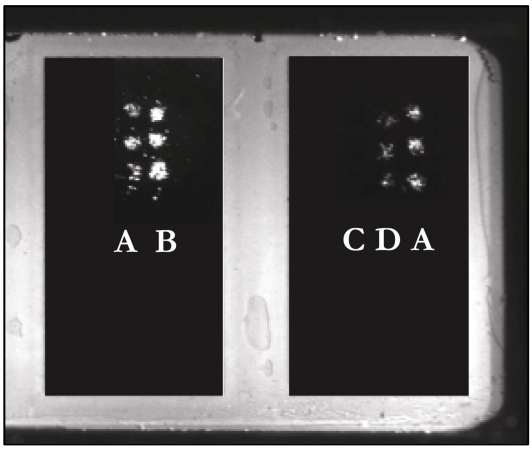

Figure 7
Reverse transfection on a 4-well Lab-Tek™ Chamber Slide™. Spotted plasmids on two wells were pBD-NFkB (B), pBD-p53 + pAD-TRAF as negative control (C) and pBD-p53 + pAD-SV40T as positive control (D) together with GAL4-pZsGreen as reporter, respectively. Autofluorescent control plasmid was pcDNA4-EGFP (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.

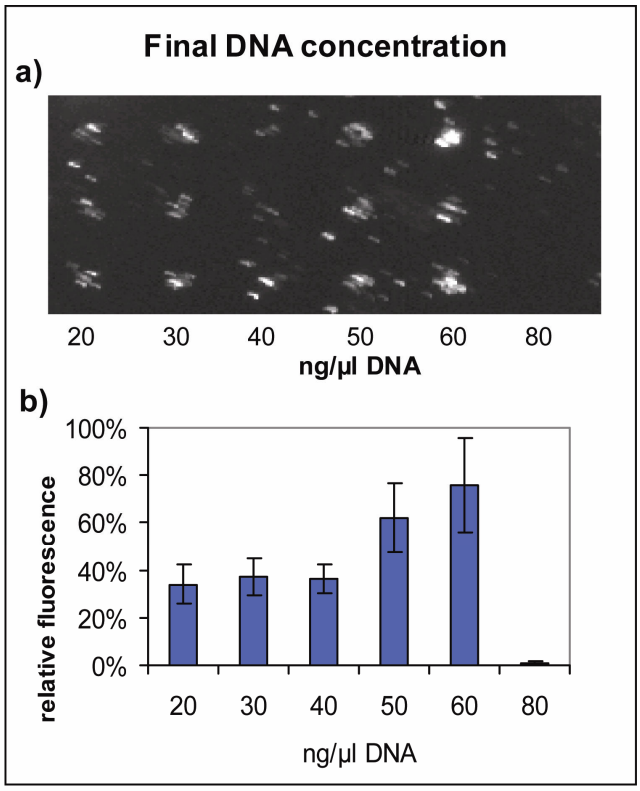

Figure 8

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.

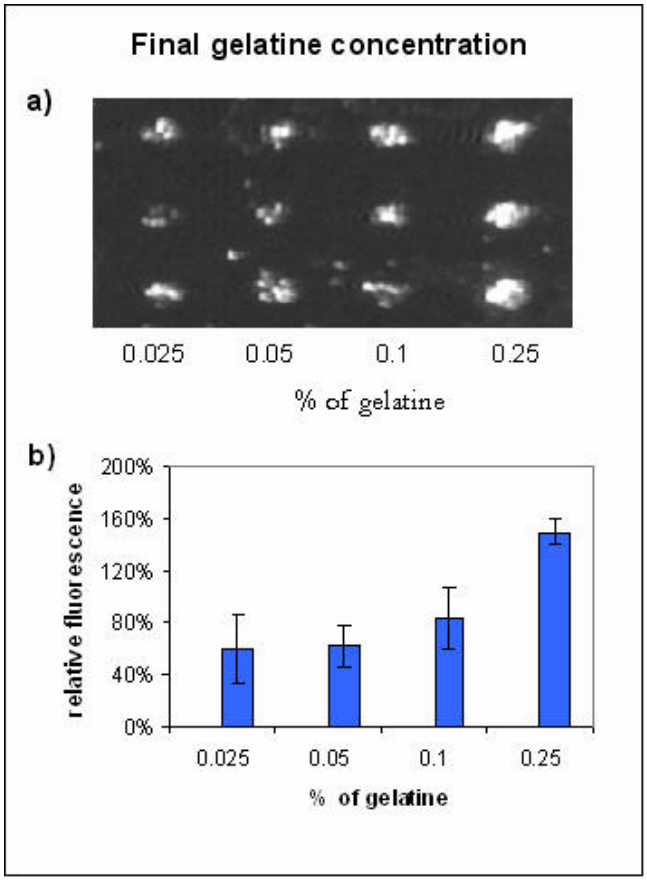

Figure 9
Different concentrations of gelatine in samples prepared by LD-method. Tested plasmids were pBD-p53 together with interacting partner pAD-SV40T and GAL4-pZsGreen as reporter. PcDNA4-EGFP was spotted as reference value for fluorescence Best fluorescent signals of the reporter protein were found 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).

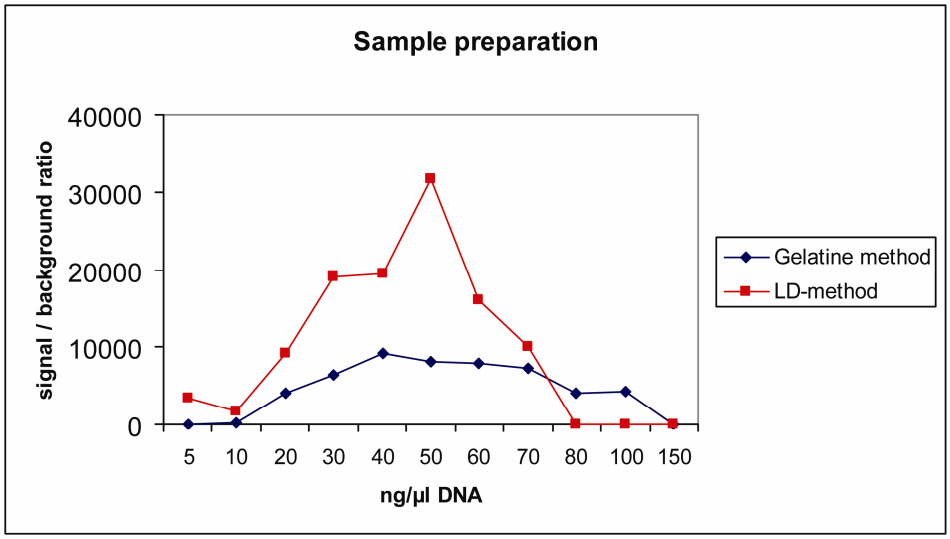

Figure 10

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.

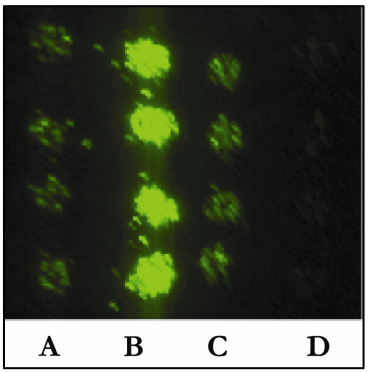

Figure 11
Expression of fluorescent ZsGreen protein in comparison with EGFP. Reporter plasmid GAL4-pZsGreen was co-transfected with pBD-NF-•B (B), pBD-p53 + pAD-SV40T (C) and pBD-p53 + non-interacting partner pAD-TRAF, where no ZsGreen was expressed (D). PcDNA4-EGFP was spotted nearby (A) as autofluorescent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

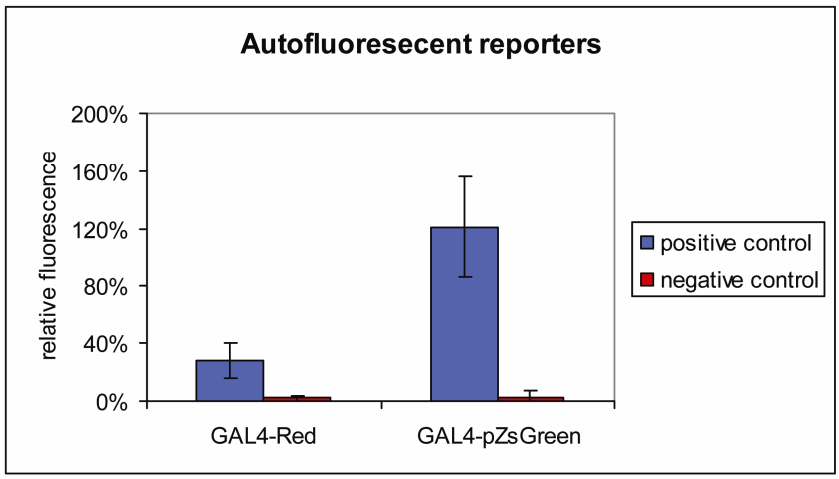

Figure 12
Signals of red reporter GAL4-Red and green reporter GAL4-pZsGreen. Expression of reporter proteins was in consequence of transfecting cells with pBD-p53 + pAD-SV40T as positive control. Nearly no reporter was expressed when cells were transfected with non-interacting pBD-p53 and pAD-TRAF as 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.

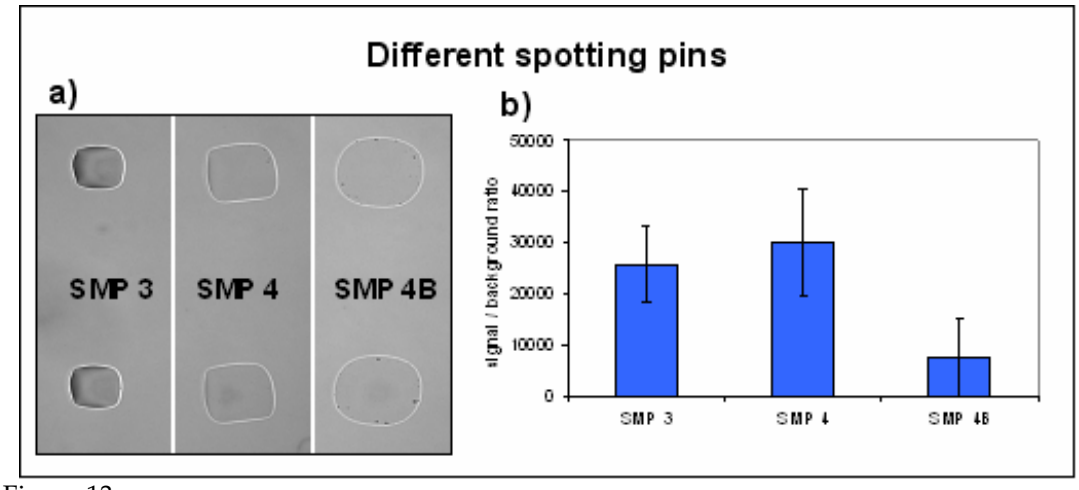

Figure 13
Comparison of different spotting pins used for automated spotting using pcDNA4-EGFP (gelatine method) as a DNA sample. First check was done before transfection with the microscope (Figure 13a). Here the SMP4B pin seems to be best. But signal intensity of EGFP fluorescence after transfection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

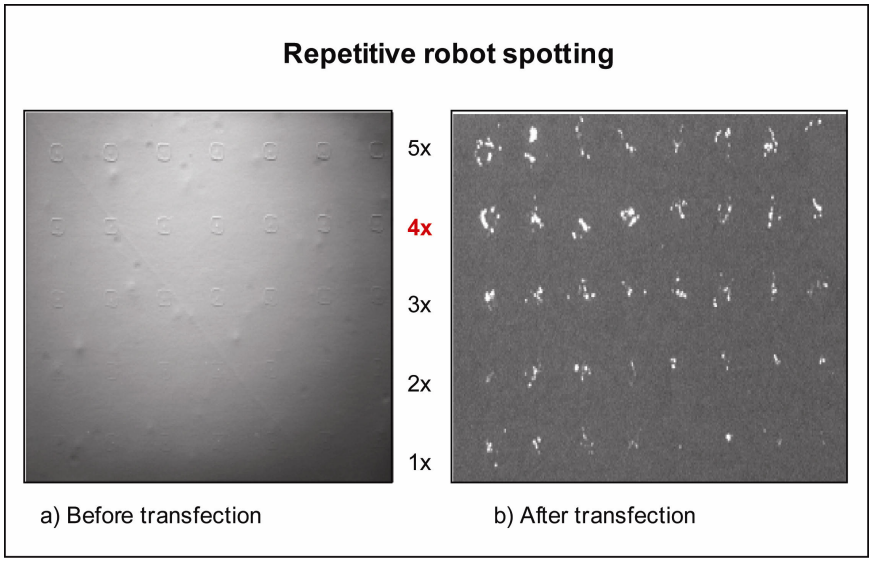

Figure 14
Repetitive robot spotting for amplification of fluorescent signal. Sample of pcDNA4-EGFP was prepared by gelatine method and spotted on slide for 1 up to 5 times on the same area with drying time in between. Before transfection, spots were checked by microscope (Figure 14a). After transfection 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.

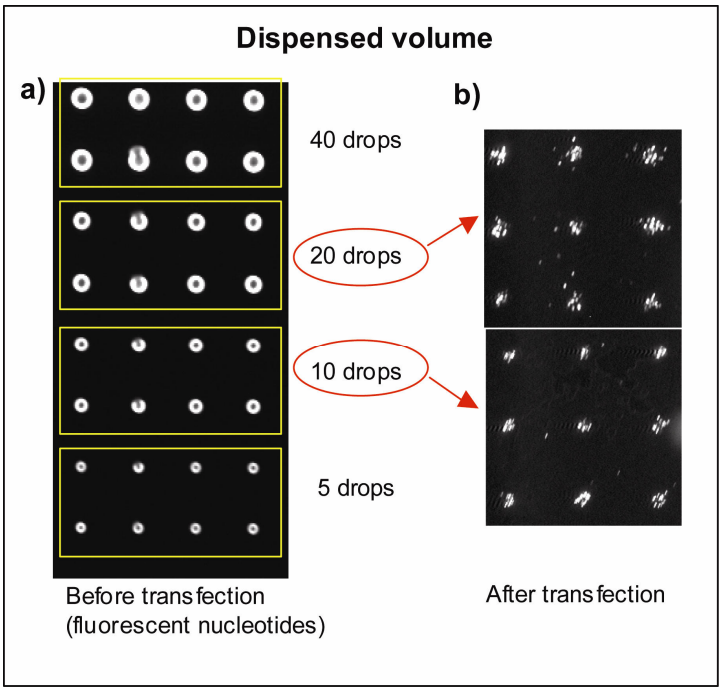

Figure 15

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.

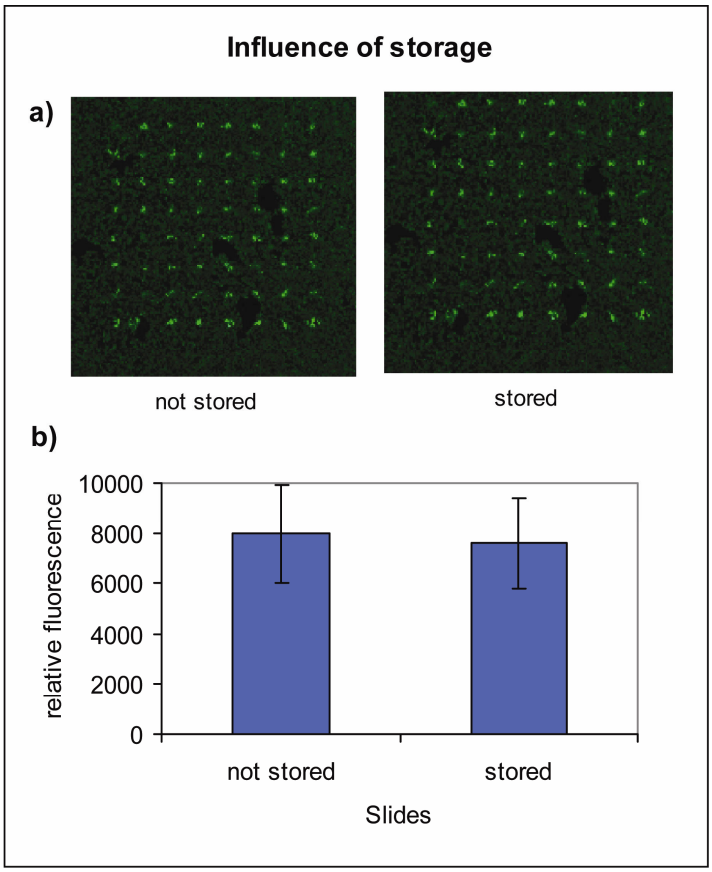

Figure 16

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.

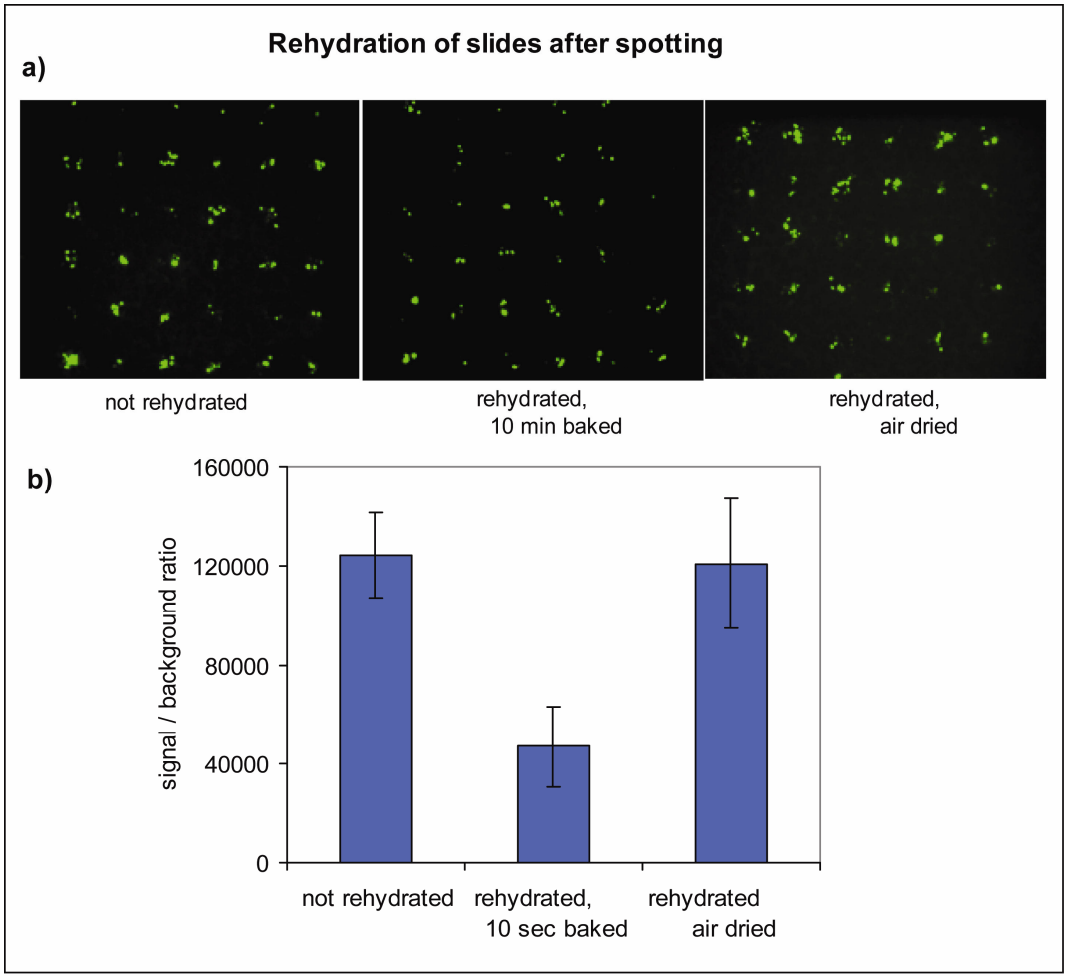

Figure 17

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.

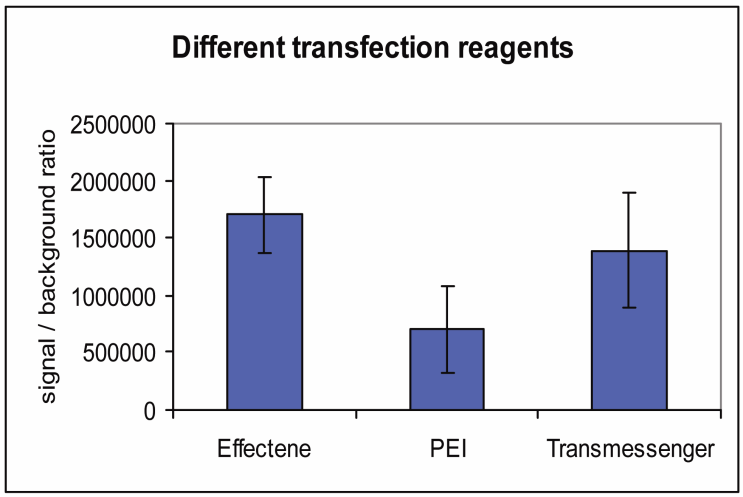

Figure 18
Comparison of different transfection reagents for reverse transfection. Graph of ratio of EGFP signals to background of non-transfected cells was best with Effectene® and a little bit lower but in the same range with TransMessenger Transfection Reagent. Reverse transfection with PEI was successful 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.

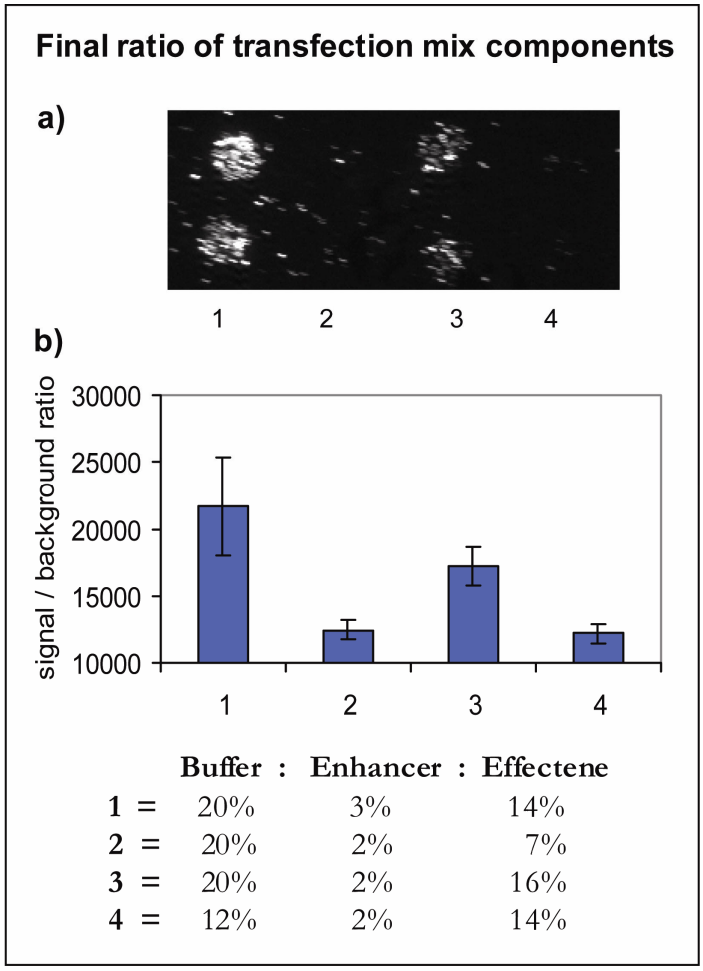

Figure 19

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.

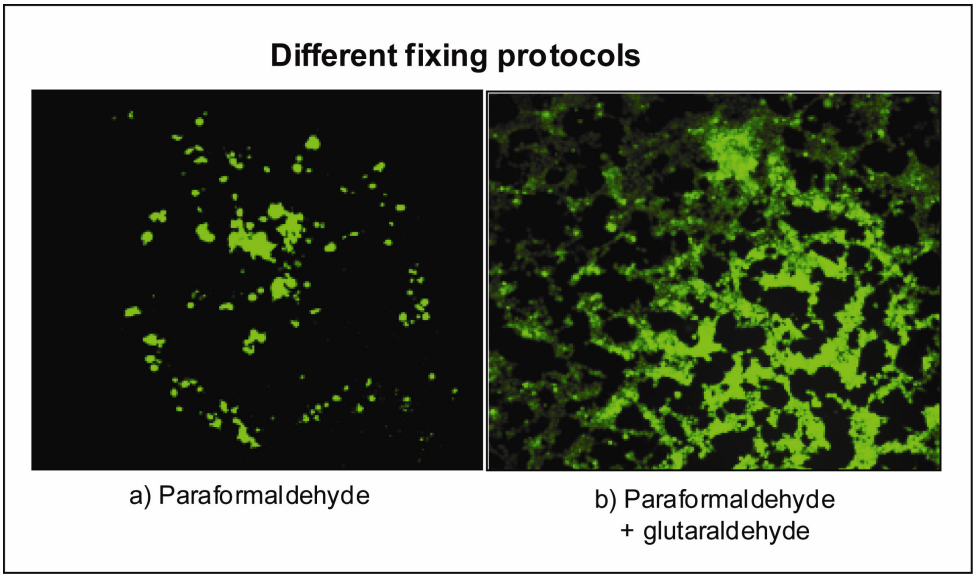

Figure 20

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.

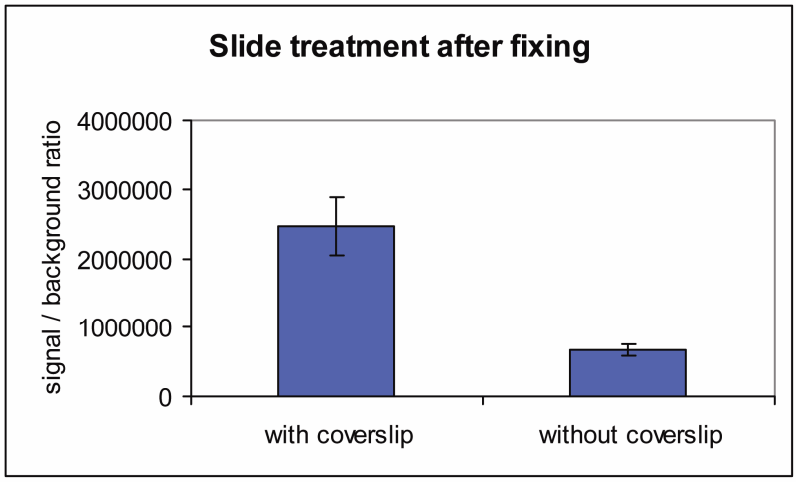

Figure 21
Different treatment of the slides after cells fixing. The intensity of the EGFP signals on slides with a coverslip was much higher than for slides without coverslip.

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.

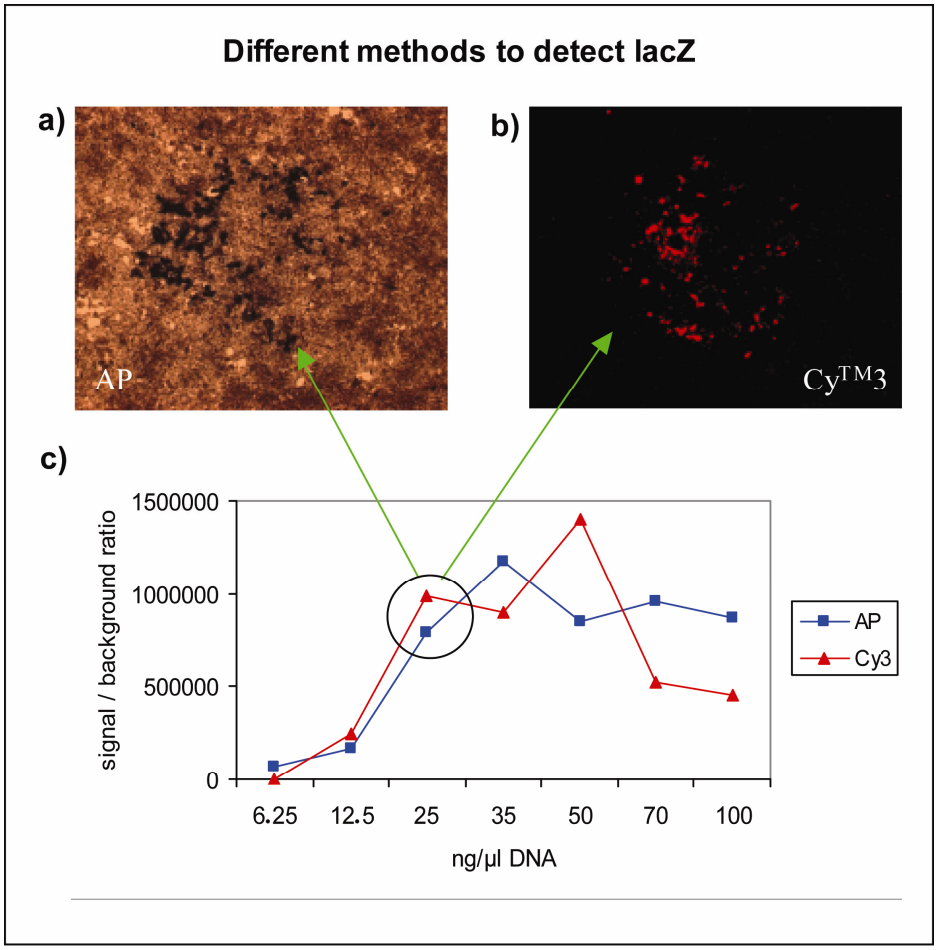

Figure 22

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.

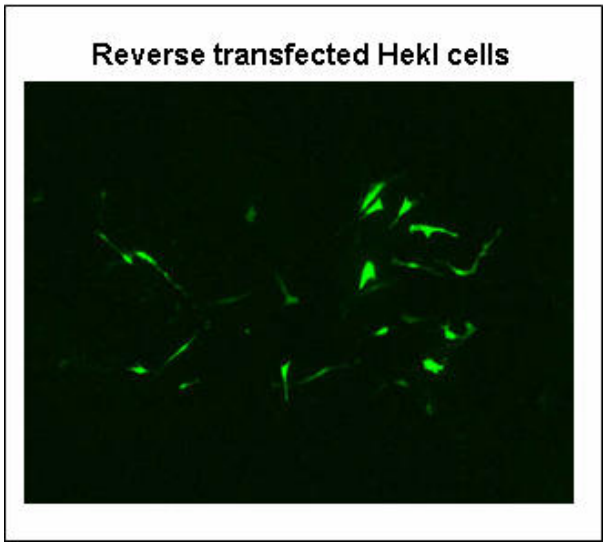

Figure 23
Reverse transfection of fibroblast cell line Hekl. The spotted sample contained pBD-NF-•B plasmid. In co-transfection with reporter plasmid GAL4-pZsGreen, cells expressed fluorescent protein ZsGreen. Image was acquired using microscope.

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).

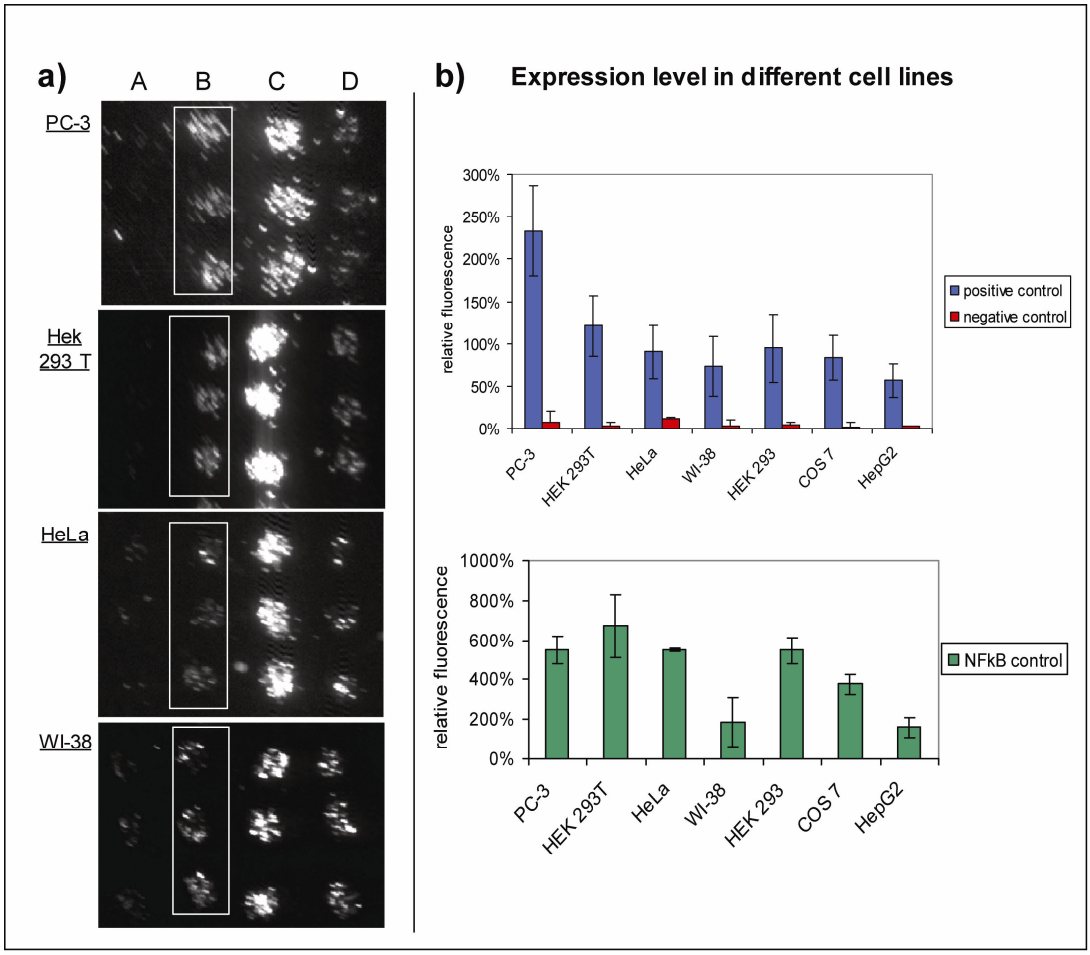

Figure 24

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-•B 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).

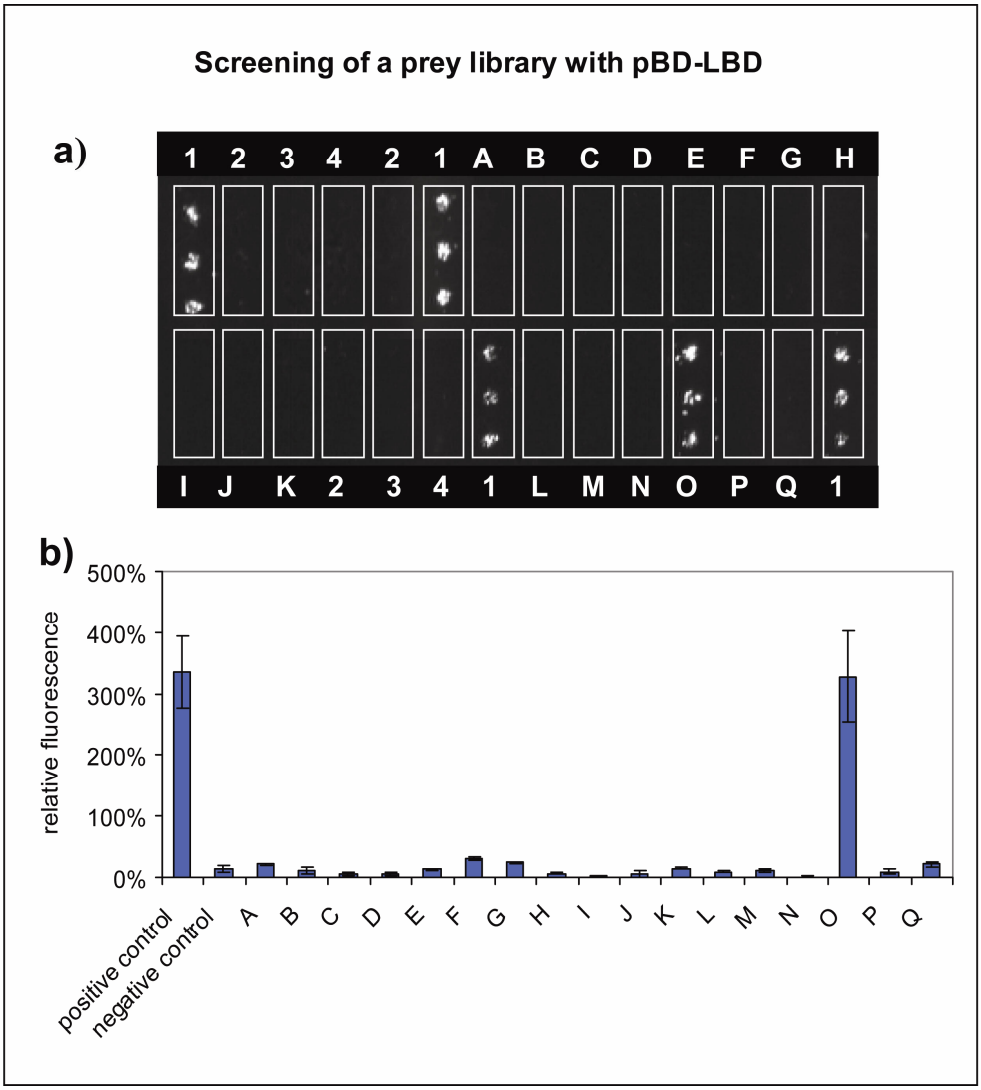

Figure 25

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).

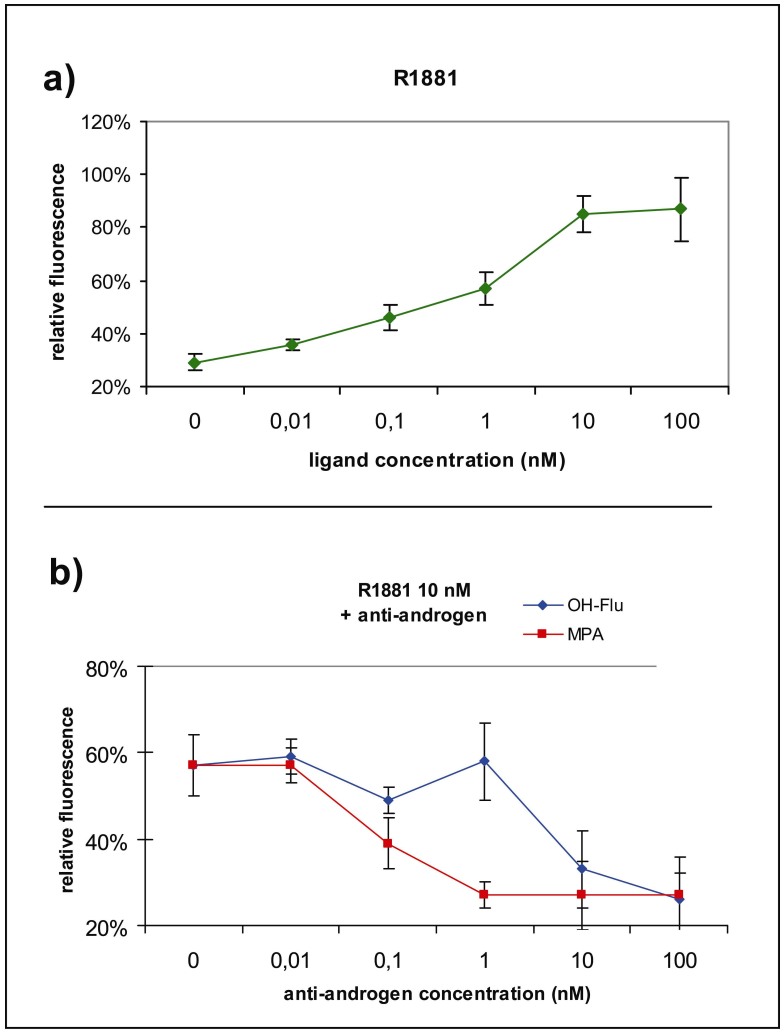

Figure 26

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.

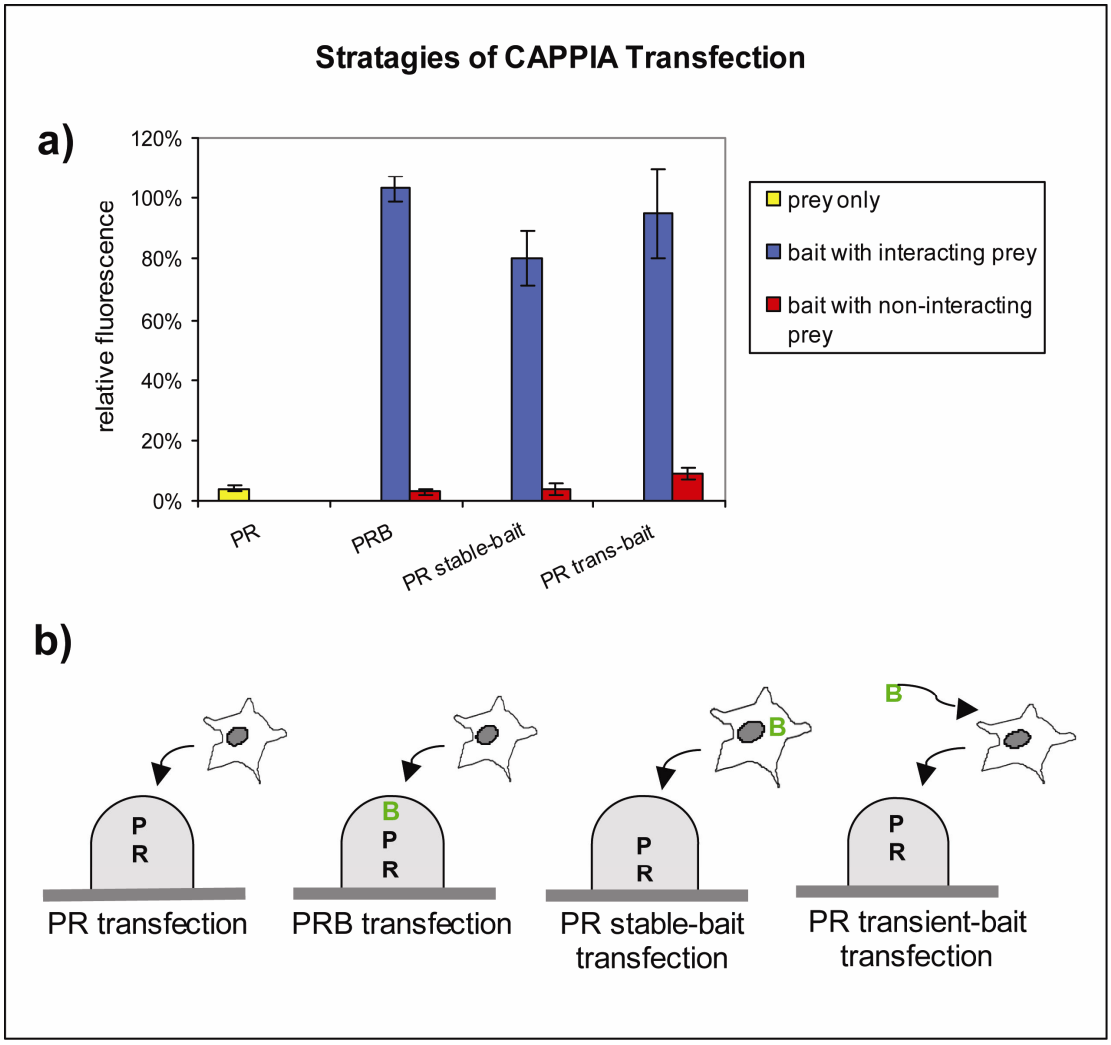

Figure 27

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.