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A CELL SPOT MICROARRAY METHOD FOR HIGH-THROUGHPUT BIOLOGY

by

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To Pia, Silvia and Sofia

My family, my joy and my motivation

ABSTRACT

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A cell spot microarray method for high-throughput biology

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High-throughput screening of cellular effects of RNA interference (RNAi) libraries is now being increasingly applied to explore the role of genes in specific cell biological processes and disease states. However, the technology is still limited to specialty laboratories, due to the requirements for robotic infrastructure, access to expensive reagent libraries, expertise in high-throughput screening assay development, standardization, data analysis and applications. In the future, alternative screening platforms will be required to expand functional large-scale experiments to include more RNAi constructs, allow combinatorial loss-of-function analyses (e.g. gene-gene or gene-drug interaction), gain-of-function screens, multi-parametric phenotypic readouts or comparative analysis of many different cell types. Such comprehensive perturbation of gene networks in cells will require a major increase in the flexibility of the screening platforms, throughput and reduction of costs. As an alternative for the conventional multi-well based high-throughput screening -platforms, here the development of a novel cell spot microarray method for production of high density siRNA reverse transfection arrays is described. The cell spot microarray platform is distinguished from the majority of other transfection cell microarray techniques by the spatially confined array layout that allow highly parallel screening of large-scale RNAi reagent libraries with assays otherwise difficult or not applicable to high-throughput screening. This study depicts the development of the cell spot microarray method along with biological application examples of high-content immunofluorescence and phenotype based cancer cell biological analyses focusing on the regulation of prostate cancer cell growth, maintenance of genomic integrity in breast cancer cells, and functional analysis of integrin protein-protein interactions *in situ*.

Keywords: High-throughput screening, RNA interference, cell microarrays

TIIVISTELMÄ

Juha K. Rantala

Solumikrosirumenetelmä solubiologisiin seulontatutkimuksiin

VTT Lääkekehityksen biotekniikka; Biokemian laitos, Turun yliopisto, Turku

RNA-interferenssin (RNAi) käyttö geenituotteiden toimintojen tutkimuksessa on vuosikymmenessä kehittynyt solubiologisen tutkimuksen merkittävimpien teknologioiden joukkoon. RNAi-menetelmät ovat mahdollistaneet myös eri solubiologisten signaalivälitysreittien kartoittamisen koko perimänlaajuisten geenitoimintojen suurtehoseulonnan avulla. Teknologian nopeasta kehityksestä ja laajamittaisesta hyödyntämisestä huolimatta, RNAi-menetelmien käyttö suurtehoseulontaan rajoittuu edelleen erikoislaboratorioihin huomattavien laite-, reagenssi- sekä tietotaitovaatimusten johdosta. Lisäksi yleisimmin käytetyt kuoppalevypohjaiset seulonta-menetelmät rajoittavat seulontatutkimusten laajuutta, eri analyysimenetelmien käyttöä sekä useiden mallisolulinjojen rinnakkaista seulontaa suuren reagenssikulutuksen takia. RNAi-suurtehoseulonnan kustannuksien ja laiteriippuvuuden alentamiseksi sekä monipuolisuuden lisäämiseksi tulevaisuudessa tarvitaan uusia vaihtoehtoisia seulontamenetelmiä. Tämän väitöskirjatyön lähtökohtana oli uuden kuoppalevyillä tehtäviä seulontamenetelmiä edullisemman ja monipuolisemman RNAi-suurtehoseulontamenetelmän kehittäminen. Tutkimuksessa kehitettiin solujen siRNA käänteistransfektioon perustuva solumikrosirumenetelmä, joka tarkasti rajattujen siRNA näytepisteiden avulla mahdollistaa jopa koko ihmisen perimänlaajuisten siRNA näytekirjastojen seulonnan yksittäisellä mittasirulla. Menetelmän suuri näytetiheys sekä sirujen pieni pinta-ala mahdollistavat myös erikoismittamenetelmien käytön suurtehoseulonnassa, jotka teknisistä tai kustannussyistä eivät sovellu käytettäväksi suurtehoseulontaan kuoppalevyillä. Väitöskirjan ensimmäinen osatyö kuvaa menetelmän kehittämistyön. Väitöskirjan toisessa, kolmannessa ja neljännessä osatyössä menetelmää on hyödynnetty syöpäbiologisissa seulontatutkimuksissa selvitettäessä geenejä jotka vaikuttavat eturauhassyöpäsolujen kasvuun, rintasyöpäsolujen solunjakautumiseen sekä integriini tarttumisreseptorien aktiivisuuden säätelyyn.

Avainsanat: RNA interferenssi, suurtehoseulonta, solumikrosirumenetelmä

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ABBREVIATIONS

AR	Androgen receptor
bp	base pair
BrCa	Breast cancer
BSA	Bovine serum albumine
cDNA	Complementary DNA
CLDN4	Claudin-4
CSMA	Cell spot microarray
CFP	Cyan fluorescent protein
DICER	Dicer 1, ribonuclease type III
DNA	Deoxyribonucleic acid
dsRNA	Double strand RNA
ECM	Extracellular matrix
esiRNA	Enzymatically prepared small interfering RNA
GFP	Green fluorescent protein
GINS2	GINS complex subunit 2 (Psf2 homolog)
GPR160	G protein-coupled receptor 160
HSPBAP1	HSPB (heat shock 27kDa) associated protein 1
HTS	High-throughput screening
ITGB1	Integrin beta-1
mRNA	Messenger RNA
miRNA	Micro RNA
NPY	Neuro peptide Y
PBS	Phosphate buffered saline
PCa	Prostate cancer
PCR	Polymerase chain reaction
PLA	Proximity ligation assay
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
TMA	Transfection microarray

LIST OF ORIGINAL PUBLICATIONS

I. **Rantala JK**, Mäkelä R, Aaltola AR, Laasola P, Mpindi JP, Nees M, Saviranta P, Kallioniemi O: A cell spot microarray method for production of high density siRNA transfection microarrays. *BMC Genomics*. 2011, 12(1); 162.

II. **Rantala JK**, Edgren H, Lehtinen L, Wolf M, Kleivi K, Moen Vollan HK, Aaltola AR, Laasola P, Kilpinen S, Saviranta P, Iljin K, Kallioniemi O: Integrative functional genomics analysis of sustained polyploidy phenotypes in breast cancer cells identifies an oncogenic profile for GINS2. *Neoplasia*. 2010, 12 (11); 877-888.

III. **Rantala JK**, Aaltola AR, Tuomi S, Mäkelä R, Laasola P, Kohonen P, Saviranta P, Kallioniemi O, Ivaska J: Comparative functional genomics analysis of endogenous protein interactions *in situ*. *Submitted*.

IV. **Rantala JK**, Björkman M, Gupta S, Saeed K, Kohonen P, Alanen K, Iljin K, Kallioniemi O: Identification of genes required for the growth and survival of prostate cancer cells in androgen-deficient conditions. *Submitted*.

INTRODUCTION

Technological revolution in cell biological research during the past decade has resulted in exponential increase of data produced in mammalian systems due to new tools allowing analysis of cellular processes in systemic scale. Among the most fundamental novel techniques described for mammalian cell biology within the last decade was the discovery and subsequent development of tools to knockdown gene functions through the use of RNA interference (RNAi) (Fire et al., 1998; Novina et al., 2004). Early on after the development of the first reagents for RNAi analysis, it was realized that RNAi lends itself also for use in high-throughput techniques. This led to the creation and evolution of commercial genome-wide RNAi reagent libraries. Following phased maturation of the technologies and instrumentation for high-throughput screening (HTS) over the past ten years, the last two-three years have brought upon the true potential of RNAi screening with multiple described studies where genome-wide query of gene functions was used for hypothesis formation. For the RNA interference gene silencing in mammalian cells, three types of small RNAs can be used. Small interfering RNAs (siRNAs) made by chemical synthesis were the first class of small RNA reagents adopted for wide-spread cell biological research use following the early applications with antisense oligonucleotides (Elbashir et al., 2001). SiRNAs are short double-stranded RNAs (dsRNA) that are typically 20-30 bp in length with 2-nucleotide overhangs on either end, including a 5′ phosphate group and a 3′ hydroxyl group. The siRNAs can be introduced into target cells by lipid transfection to cause transient sequence-specific silencing of a target gene. When introduced to the cells, the siRNA molecules are processed by a ribonuclease enzyme known as DICER (dicer 1, ribonuclease type III) and members of the Argonaute (Ago) family of proteins (Okamura et al., 2008; Kim et al., 2009). One strand of the siRNAs functions as the template for the RNA-induced silencing complex (RISC), which pairs to, and catalyzes the rapid cleavage of the mRNA with a complementary sequence. Alternatively to siRNA, short hairpin RNAs (shRNA) mimicking the micro RNA (miRNA) concept of transcriptional attenuation can be used for RNAi gene silencing (Paddison et al., 2002). ShRNAs are constructed in a plasmid backbone containing a RNA polymerase III (RNA pol III) promoter and can be lipid transfected or transduced in target cells using virus vectors containing the packaged shRNA. ShRNA transduction results in the stable integration and expression of the shRNA in the target cells (Brummelkamp et al., 2002). In comparison to the 20-30 nucleotide siRNA molecules, shRNAs are initially produced as single-stranded molecules of 50–70 nucleotides in length which form a stem–loop structure *in vivo* through base pairing of the complementary 19–21-nucleotide stem sequences of the molecules. In cells,

the expressed shRNAs exit the nucleus and are recognized and cleaved at the loop by the ribonuclease DICER releasing the dsRNA stem sequence as a siRNA molecule which can be incorporated in the RISC to active target gene silencing similarly as the synthetic siRNA molecules (Meister et al., 2004). In addition to the synthetic siRNA-transfection or shRNA transduction-based techniques, enzymatically prepared siRNAs (esiRNAs) can also be used for RNAi (Luo et al., 2004; Sen et al., 2004; Shirane et al., 2004). EsiRNAs are siRNA molecules generated by endoribonuclease cleavage of long dsRNAs. For production of esiRNAs, target gene cDNAs are first amplified by PCR with primers containing a T7 polymerase specific promoter sequence, and then transcribed *in vitro* to produce long dsRNAs. The produced dsRNA molecules are digested with recombinant RNase III to produce a pool of siRNA molecules, which are subsequently purified using an affinity column (Kittler et al., 2004). The advantage of the esiRNA approach is that siRNAs against the entire coding sequence of the target gene are used for knockdown. The resulting disadvantage of esiRNA pools is that the siRNA sequence diversity per gene may cause a variety of unwanted off-target effects.

As transfection of human cells with the chemically synthesized siRNAs or shRNAs is an easy way to silence a gene of interest, RNAi has become a routine tool of substantial importance in cell biological research. High-throughput screening (HTS) of cellular effects of RNA interference libraries is also now being applied with increasing pace for gene function discovery and exploration of the roles of genes in specific cell biological processes. Despite the significant development of the RNAi reagents, methods and instrumentation, the high-throughput application of RNAi is still limited to specialty laboratories with access to robotic infrastructure and the expensive reagent libraries, expertise in HTS assay development, standardization, data analysis and applications. One of the main technical bottlenecks for RNAi-HTS is the microplate based assay platforms where most described systemic-scale RNAi studies have been performed. Due to the high reagent demand of these platforms, screening experiments in microplates are often restricted in scale and variety of available applications. In the future, alternative techniques will be required to increase availability and versatility of HTS-RNAi experiments and decrease screening costs. Robust low-cost techniques will be of critical importance also for expanding functional large-scale screens to include more RNAi constructs, alternative assay techniques and different cell types including primary cells.

The transfection microarray (TMA) technology has been proposed as a new platform for large-scale RNAi analyses allowing significant increase in experiment throughput and reduction in screening costs. Although several groups worldwide have reported various modifications of the technique, the true potential of these techniques remains to be resolved. Here, the development of a novel miniaturized cell spot microarray

(CSMA) method which facilitates utilization of the transfection microarray technique for disparate RNAi analyses is described. To promote rapid adaptation of the method, the concept has been tested with several different cell biological assay types and cell lines providing proof for the versatility and reproducibility of the method in the systematic screening of gene functions in cultured human cells.

REVIEW OF THE LITERATURE

1. RNA interference screening

During the 'post-genomic era' of cell biological research, the scope and diversity of functional cell based analyses has moved onto a level unimaginable only a decade ago. The information produced by the genome sequencing efforts of yeast (*Saccharomyces cerevisiae*), fruit fly (*Drosophila melanogaster*), roundworm (*Caenorhabditis elegans*) and human has led to development of genome covering reagent collections of both cDNA and siRNA (shRNA) libraries which have transformed the nature of biological inquiry allowing parallel organism-scale functional explorations. This has also led to the establishment of a whole new industry of biological reagent production along with new high-end analysis instrumentation to compensate for the substantial increase of samples included on cell biological experiments. Adaptation of RNA interference (RNAi) to laboratory practice for loss-of-function genomic studies is among the most fundamental novel techniques emerging following sequencing of the genomes. RNAi altered the concept of experimental approaches used to study gene functions *in vitro* and led to the rapid and widespread use of the technique in all areas cell biological research. *Caenorhabditis elegans* and *Drosophila melanogaster* were the first model organisms where it was originally recognized that RNAi can be exploited to suppress gene expression (Fire et al., 1998; Kennerdell and Carthew, 1998). These organisms served also as the first models for systematic loss-of-functions RNAi screens, where highly parallel RNAi was used for the rapid identification of gene functions in many biological processes (Fraser et al., 2000; Piano et al., 2002; Kamath et al., 2003; Kiger et al., 2003; Lum et al., 2003; Porthof et al., 2003; Bettencourt-Dias et al., 2004; Boutros et al., 2004; Echard et al., 2004). The great success of already these first described RNAi screens facilitated significantly the development of comprehensive genome-scale RNAi reagent libraries which thereafter rapidly became accessible for academic laboratories around the world, and at the same time, allowed design and completion of more complex loss-of-function screens (Björklund et al., 2006; Kondo and Perrimon 2011).

Soon after the description of RNAi in the initial model organisms, it was discovered that RNAi also suppresses gene expression in mammalian cells (Elbashir et al., 2001). This led to intense research efforts to resolve the molecular mechanisms of RNAi in human cells followed with development of highly sophisticated algorithms for design of chemically synthesized siRNAs for targeting human genes. Thereafter, the screening of commercial siRNA libraries has become an essential tool of biological studies to elucidate gene functions in human cells as well. In a typical mammalian RNAi screening

experiment, cultured cells are dispensed onto wells of 96- or 384-multiwell format plates. SiRNA samples mixed with lipid transfection agent are applied onto the wells and cells are exposed to the reagents for 48 to 72 hours to allow RNAi mediated target gene silencing. Following the siRNA transfections, cells are analyzed for a phenotype of interest, such as cell viability or growth characteristics, morphology or for example reporter gene assays. Cancer research has been in the forefront of RNAi screening in human cells from the beginning. Large scale screens using synthetic siRNAs targeting defined gene families or the complete genome have been used for cancer research to elucidate multiple biological processes from a systemic scope. Among the first large-scale human RNAi screens, synthetic siRNAs were used in HeLa cervical cancer cells to identify modulators of TRAIL-induced apoptosis (Aza-Blanc et al., 2003) and regulators of chemoresistance (Mackeigan et al., 2005), whereas U-2-OS sarcoma cells were applied in a genome-wide cell cycle regulation screen (Mukherji et al., 2006). Outside the cancer context, analysis of kinases involved in endocytosis through clathrin and caveolae-mediated pathways (Pelkmans et al., 2005) was among the first described screens using synthetic siRNAs. After the early applications the complexity of siRNA screen designs evolved significantly including through application of more complex assay techniques (Kolas et al., 2007) and combination of e.g. siRNA loss-of-function screening with gain-of-function approaches (Varjosalo et al., 2008). In the more recent screens, synthetic siRNAs have been applied for studies in multiple different fields of cell biology, including detailed characterisation of regulation of cell cycle (Kittler et al., 2007; Fuchs et al., 2010), cell adhesion (Winograd-Katz et al., 2009), neurite growth (Loh et al., 2008), drug resistance (Iorns et al., 2008; Turner et al., 2008), circadian clock (Zhang et al., 2009), DNA damage responses (Paulsen et al., 2009), anchorage-independent growth of cancer cells (Irie et al., 2010) and many more.

As many mammalian cell types, including primary human cells, are often considered resistant to or hard to transfect using conventional lipid based siRNA transfections methods, a need for more efficient ways to introduce siRNAs into cells emerged. The alternative approach to transduce mammalian cells with viruses containing packaged shRNA expression plasmids for RNAi, has been shown to provide highly effective gene suppression in a variety of mammalian cell types (Abbas-Terki et al., 2002; Brummelkamp et al., 2002; Paddison et al., 2002; Stewart et al., 2003, Siolas et al., 2005). In the early large-scale screens using shRNA-expressing retroviral vectors in human cells, pools of retroviruses were used for identification of components of the p53 pathway (Berns et al., 2004). Thereafter shRNA-expressing retroviral plasmids were used for identification of genes involved in proteasome functioning (Paddison et al., 2004; Silva et al., 2005), tumor-suppressor genes (Kolfschoten et al., 2005; Westbrook et al., 2005) and regulation of cell cycle and mitotic progression (Moffat et al., 2006). In more recent studies, shRNA libraries have also been applied for analysis of cell motility (Simpson et al.,

2008), drug resistance and -sensitivity (Mullenders et al., 2009a; Fotheringham et al., 2009), circadian clock (Maier et al., 2009), p53 functions (Mullenders et al., 2009b), neurite growth (Ossovskaya et al., 2009) and novel tumor suppressor genes (Gobeil et al., 2008). Among the most active fields in shRNA based RNAi screening has been the DNA damage response research with multiple systemic-scale screens described thus far (Hurov et al., 2010; Lovejoy et al., 2009; O'Connell et al., 2010; Smogorzewska et al., 2010).

The number of reported high-throughput screens using the siRNA and shRNA as well as the esiRNA (Ding et al., 2009; Slabicki et al., 2010) reagent libraries is now expanding with increasing pace. While these reports establish the precedent that RNAi screening using the conventional techniques can be employed to perform loss-of-function screens in a variety of cell contexts, it is clear that further development on the performance and limitations of the screening platform is required before the large-scale RNAi applications become widely available also to small research laboratories. Moreover, most described large-scale RNAi studies have relied on the analysis of a single parameter at a time. Though well suited for specific biological questions, this provides a restricted view on cell biology. Furthermore, many assays cannot be converted to HTS format due to methodological limitations or cost considerations. In the future, significantly more flexible screening platforms will be required to expand functional large-scale screens to include more RNAi constructs, allow combinatorial siRNA analyses and application of multiplexed high-end assay techniques.

2. Transfection cell microarrays

From the very start of RNAi screening, the costs of the large RNAi reagent libraries, requirements for excessive instrumentation and automation, expertise in HTS assay development, standardization, data analysis and application knowledge has limited the general accessibility of large-scale RNAi screening. Miniaturization of the screening platforms to microarray formats has been suggested as an alternative for multiwell based RNAi screening approaches to provide an economical and robust way to systematically screen the genome. During the past ten years, several groups have provided proof that mammalian RNAi can be adapted for use in different transfection cell microarray formats. The transfection cell microarrays are miniaturized array-based screening platforms that depend on micro-patterning of RNAi reagents on a cell culture surface. With simplification, a transfection cell microarray is a cell culture surface, such as a glass microscope slide, where e.g. siRNA samples and lipid transfection agent are deposited in a microarray patterned format. Commonly, a microarray printer is used to deposit the samples onto the array surface. For transfection of cells, the siRNA-lipid

microarrays are covered with cells in culture medium for up-take of the reagents (cDNA, siRNA or shRNA) from the arrayed spots (Figure 1.). Following commonly 48 to 72 hour exposure to the printed samples, cells growing on the arrays are fixed and prepared for microscopic analysis. Use of all the three approaches for mammalian RNAi (siRNA, shRNA and esiRNA), have been reported compatible with the cell microarray format (Mousses et al., 2003; Wheeler et al., 2005; Bailey et al., 2006).

2.1. Reverse transfection

The primary technique common for majority of the transfection cell microarray techniques is lipid based transfection of the cells with the so-called reverse transfection method. The first description and utility of reverse transfection with mammalian cells was reported in 2001 (Ziauddin and Sabatini, 2001). This study demonstrated the down-scaling of high-throughput gene function analysis to the microarray level with a proof-of-principle screen of cDNA plasmid-expression vectors for target gene over-expression in mammalian cells. 192 individual cDNA expression plasmids in an aqueous gelatin solution were deposited onto amino-silane coated glass slides using a contact microarray printer. The dried slides were exposed to lipid transfection reagent, placed in a cell culture dish, and covered with high density suspension of adherent HEK293T cells in growth medium. The cells were cultured over the array surface for 40 hours resulting in localized expression of the target genes on top of the cDNA samples after which the arrays were fixed and cells prepared for immunofluorescence analysis. The

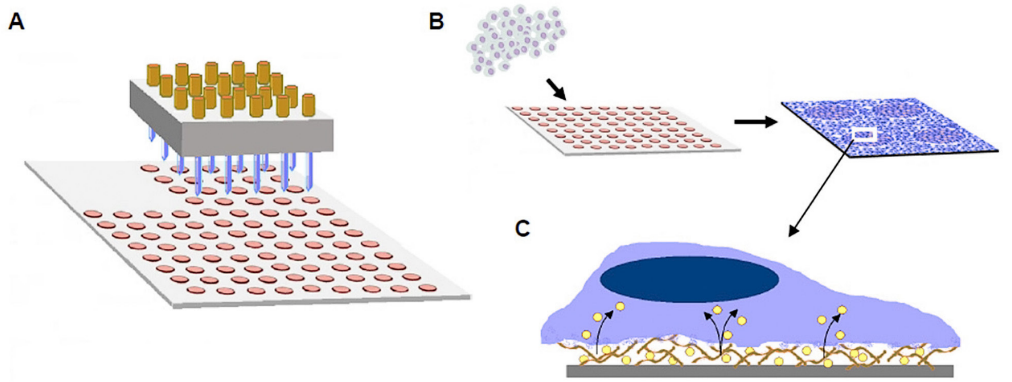

Figure 1: Principle of the transfected cell microarrays. (A) cDNA or siRNA samples are printed onto a glass or plastic cell culture surface using a robotic microarray printer. The printed samples are complexed with lipid transfection agent on the surface or prior to the printing. **(B)** Adherent cells in uniform cell suspension are dispersed over the array surface in growth medium and allowed to adhere on the array surface. **(C)** siRNA-lipid micelles dissolving from the array surface results in localized transfection of the cells, termed reverse transfection. The figure is modified from Rantala et al., 2011.

concept was tested also with HeLa and A549 lung cancer cells in the same study. The procedure for creating microarrays of transfected cells was thereafter called transfected cell microarray technique and the transfection method was named reverse transfection. In contrast to conventional transfection protocols where transfection reagents are added on top of cells, here the expression constructs were deposited first and the cells were dispensed second on top of the reagents.

In the original description of cell microarrays for reverse transfection, the cDNA samples were complexed with the transfection agent on top of the array surface. Alternatively, cDNA and the transfection agent could also be complexed readily before array printing and printed to the slide in a mixture (Figure 1.). Both methods used for complexion of the cDNA samples and the lipid agent were successfully used to create microarrays of cell clusters simultaneously transfected with different cDNA plasmids demonstrating the potential of this miniaturized method for gain-of-function genomics analyses. Moreover, the success of the reverse transfection method provided evidence that it could lend itself also for use with siRNA reverse transfection as described next.

2.2. Transfection microarray variations

Since the initial transfection microarray report for cDNA reverse transfection of human cells, several research groups have developed and reported proof-of-concept cell microarray techniques for alternative applications, including RNAi analysis in mammalian cells. The original cDNA transfection method has later been applied in five different studies. In the first, the approach was used for analysis of overexpression of G-protein-coupled receptors (GPCR) in HEK293T cells (Mishina et al., 2004). Here, the arrays were prepared on bottom of 96-well microplates in comparison to the glass slides used originally. 36 individual expression constructs were deposited per 96-well and complexed with the transfection agent on the surface according to the original method. The cDNA transfection microarray method was thereafter described also in an analysis of transcriptional regulation by cAMP-dependent protein kinase in SH-SY5Y cells (Redmond et al., 2004), apoptosis inducing proteins in HEK293T cells (Palmer et al., 2006), characterization of proteins coded from the human chromosome 21 (Hu et al., 2006), and in analysis of Human Herpesvirus 8 (HHV8) coded genes impacting on the activation of the cellular transcription factor nuclear factor-kappa B (NF-κB) (Konrad et al., 2009). In all the four latter reports, the arrays were prepared on glass slides according to the original protocol. An alternative approach taking advantage of the same transfection protocol, but based on a more advanced surface chemistry, described preparation of patterned transfection cell arrays on micropatterned glass surfaces with self-assembled monolayers (SAM) of alkanethiols (Yamauchi et al., 2004). In this study the authors used gold-evaporated glass slides as substrate for photolithographic micropatterning of self-

assembled monolayers of alkanethiols for immobilization of cDNA-lipid samples and cells. Reverse transfection of mammalian cells using the technique was demonstrated with eGFP cDNA transfection and expression in HEK293T cells.

The first described cell microarray application with siRNAs described an RNAi reverse transfection method for murine and HeLa cells (Kumar et al., 2003) (Figure 2.). In the study the authors used arrayed cDNA expression constructs for eGFP (enhanced GFP) fused to *MYOD1* (myogenic differentiation 1) as a reporter for measuring RNAi knock-down efficiency. Different siRNA and shRNA reagents targeting MYOD1 were co-printed with the cDNAs on amino-silane coated glass slides and complexed on the spots with lipid transfection agent for knock-down analysis in HeLa cells. The authors used this method to evaluate efficacy of different siRNA constructs for gene silencing providing the initial description and rationale for the concept of validation of siRNA efficiency as basis for accurate high-throughput RNAi screening.

The second description of siRNA based cell microarrays reported a similar method with focus on establishing conditions for high-throughput RNAi screening experiments (Mousses et al., 2003). Here, arrayed fluorescent rhodamine-labeled siRNAs targeting eGFP were complexed with a lipid transfection agent and printed onto poly-lysine coated microscope glass slides as complex to demonstrate potent siRNA uptake and localized knock-down of eGFP expression in HeLa cells. This report was the first application where spatially localized effects of RNAi in the microarray format were successfully demonstrated. In subsequent reports, RNA interference microarrays were demonstrated in several mammalian cell types (Silva et al., 2004) and also in *Drosophila* cells (Wheeler et al., 2004). Whereas these early siRNA transfection microarray reports described the application of the original reverse transfection method for RNAi analysis using proof-of-concept experiments with reporter gene assays, the first report of silencing endogenous targets was reported with knock-down analysis of β-subunit of the coat protein complex (COPI) in four different human model cell lines (Erfle et al., 2004) without the use of an exogenous reporter (Figure 2.). Here, the siRNA samples were complexed with two different transfections agents prior to printing and printed onto untreated glass microscope slides equipped with a chamber gasket to allow culture of the cells on top of the arrays.

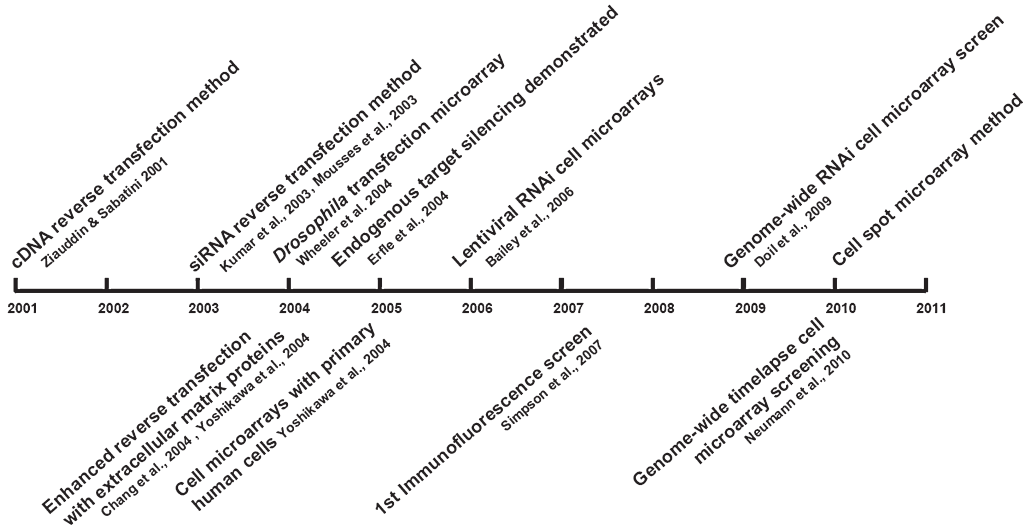

Figure 2: Timeline of the development and hallmarks of the transfected cell microarray techniques. The initial description and launch for the development of transfected cell microarray methods was described in 2001 (Ziauddin & Sabatini 2001). The first siRNA cell microarray reports were published in 2003 (Kumar et al., 2003; Mousses et al., 2003).

Common to all of the above described cell microarray techniques, the arrays are based on a uniform lawn of cells grown over the array surfaces. These so-called cell carpet approaches were also used in reports where clusters of transfected cells were analyzed for pro-apoptotic genes (Mannherz et al., 2006), cell cycle regulators (Neumann et al., 2006 & 2010; Hutchins et al., 2010), components of the mammalian secretory machinery (Simpson et al., 2007), cellular cholesterol regulation (Bartz et al., 2009) and DNA repair proteins (Doil et al., 2009).

Expanding the technical scope and compatibility of the cell microarrays to alternative applications, several groups have reported also different techniques for preparing patterned transfection microarrays. Among the first reports with an alternative transfection technique, a method based on a specialized matrix-surface transfection termed ‘surfection’ was described (Chang et al., 2004). In the method a silicone mask was used to allow patterned coating of poly-lysine treated glass surfaces with gelatin-polyethylenimine or collagen I -polyethylenimine solutions. Cells in growth medium supplemented with cDNA expression plasmid or shRNA expression vectors were added onto the ‘microwells’ to allow target gene expression or RNAi silencing. This study was the first description for using matrix proteins to enhance the efficacy of reverse transfection in several different mammalian cell types. Following this, two subsequent reports described comparative analysis of the impact of different extracellular matrix proteins on the efficacy of reverse transfection based siRNA transfection in human

and rat cells (Yoshikawa et al., 2004; Uchimura et al., 2005) (Figure 2.). Here, the transfection cell microarray method was first described also for use with primary human cells (Yoshikawa et al., 2004) providing evidence that extracellular matrix proteins could be used to activate endocytosis processes enhancing the uptake of synthetic siRNAs in reverse transfection methods.

Expanding the scope of RNAi techniques compatible for use with the transfection microarray methods, a proof-of-concept study reported application of lentiviral shRNA vectors for array based gene silencing (Bailey et al., 2006). HEK293T cells were tested for lentiviral transduced RNAi gene silencing of exogenous reporter genes as well as endogenous targets. This study described successful transduction of lentiviral shRNAs in microarray clusters of human cells, but no additional reports using the lentiviral cell microarrays have been reported. Expanding cell microarrays to suspension cells, the first and only report for using suspension cells with siRNA transfection microarrays described an approach where oleyl poly(ethylene glycol)ether-modified glass surfaces was used for immobilization of RNAi reagents and non-adherent leukemic K562 cells (Kato et al., 2004). Spotted samples of synthetic siRNAs complexed with lipid transfection agent were shown to result in spatially confined gene silencing in the non-adherent cells.

Whereas all of the above described siRNA or shRNA based transfected cell microarrays and the lentiviral microarray approach rely on the cell carpet approach, few groups have reported also variations of cell microarrays aiming on a spatially confined array layout where the transfected cell clusters are not surrounded by non-transfected cells. A method based on cell droplets cultured on a glass surface was among the first reports with confined cell spots (Schaack et al., 2005). Here, HeLa cells suspended in growth medium supplemented with the siRNA and transfection agents were deposited as 100nL droplets on a glass slide patterned with a perfluorosilane coating using a piezoelectric dispenser for culture and siRNA transfection. The second report for a technique based on spatially confined cell spots described preparation of patterned cell culture surfaces by chemical or photolithographic etching of cell repellent poly(vinyl alcohol) or hydrogel films on a glass surface (Peterbauer et al., 2006). The method was demonstrated for reverse transfection of human and rat cells according to the original (Ziauddin and Sabatini 2001) procedure with cDNA-lipid transfection. A parallel report described use of the self-assembled monolayers of alkanethiols described originally with cDNA transfection (Yamauchi et al., 2004) also for preparation of siRNA transfected cell microarrays (Fujimoto et al., 2006). This technique was later described also in combination with an electroporation based siRNA transfection microarray method (Fujimoto et al., 2010).

2.3. Biological applications of transfection cell microarrays

Most of the reported alternative transfection cell microarray techniques are proof-of-concept studies that have focused on establishing conditions for the reverse transfections with reporter gene assays. In many cases no further applications following the original report of the techniques have been published and the compatibility of the methods for parallel knock-down experiments with larger collections of siRNA or shRNA libraries for endogenous target genes remains to be demonstrated.

Considering the applicability of these methods for high-throughput screening applications, there are three common features limiting the success of virtually all of the transfection microarray methods. 1: These techniques are often limited by the difficulty of up-scaling production of arrays with large number of siRNAs (or shRNAs). In all of the described techniques the individual arrays are comprised of a modest number of samples and multiple arrays have to be combined for the analysis to achieve larger sample coverage. 2: The methods have been designed within a specified experimental context with validated reagents and pre-validated assays and functionality of the techniques with different assays has not been tested. Moreover, in most cases only one or a few model cell lines have been included in the method optimization process and successfully used with the method. 3: The third difficulty with transfected cell arrays is the need for high-end microscopy and image analysis regimen to allow robust imaging and analysis of hundreds or thousands of cell spots at sufficient accuracy and resolution. In addition, in the cell carpet approach the non-transfected cells surrounding the clusters of transfected cells make accurate quantification of the affected cells difficult without exogenous reporter techniques such as co-transfection of fluorescent marker proteins for transfection quantification.

The only described RNAi transfection cell microarray technique partially overcoming these limitations and successfully used in more than two separate cell biological studies and moreover, the only method used for true high-throughput screening experiments targeting endogenous targets is based on the siRNA transfection microarrays in glass chamber slides (Erfle et al., 2004). In this method siRNA stock solutions pre-incubated with Lipofectamine 2000 (Invitrogen) lipid transfection agent are mixed with buffer solution containing gelatin and/or fibronectin and sucrose (Erfle et al., 2007a & 2007b). The mixed samples are deposited onto untreated chamber glass microscope slides with a coverslip thickness bottom using robotic contact microarray printer. Sample spots of roughly 300 to 400µm diameter are printed in density of 96 to 600 spots per microscope slide. For transfections a cell suspension is dispensed on top of the arrays resulting on a sub-confluent layer of cells covering the whole array surface. For RNAi screening the arrays have been imaged with timelapse microscopy for kinetic assaying of living cells or fixed and prepared for immunofluorescent staining and image analysis

(Matula et al., 2009). The first proof-of-concept study demonstrating use of this cell microarray platform for fluorescence microscopic RNAi analysis reported analysis of proteins that are involved in the endoplasmic reticulum (ER) to plasma membrane transport of proteins (Simpson et al., 2007). A temperature-sensitive variant of VSV-G protein (Vesicular stomatitis virus G glycoprotein), tsO45G, tagged with a fluorescent reporter protein CFP (cyan fluorescent protein) was expressed in HeLa cells and used as a marker to study ratios of cytoplasmic tsO45G to membrane secreted tsO45G detected by immunofluorescence following the siRNA transfection. This study indicates the feasibility of using a microscopy approach for combining an exogenous reported construct and immunofluorescence methods to systematically analyze cellular functions with RNAi cell microarrays. In the study the arrays covered though only siRNAs for 37 genes making the experiment borderline to be considered a high-throughput experiment. Expanding significantly the scale of sample coverage in cell microarray screens to genomic-scale, the second screening application with the method reported screening of genes mediating focal accumulation of endogenous P53BP1 (p53 binding protein 1) protein on chromatin in U-2-OS sarcoma cells (Doil et al., 2009). Here, the U-2-OS cells were transfected with multiple array slides each containing 384 spotted siRNA samples for 3 days after which the cells were fixed and immunostained with an antibody against P53BP1. The arrays were imaged with an automated fluorescence microscope and the spontaneously occurring nuclear foci of P53BP1 were quantified with image analysis software resulting in identification of RNF168 as a new factor that regulate the affinity of P53BP1 to chromatin. The third fluorescence microscopy based application of the same technique described RNAi quantification of genes impacting on the cellular cholesterol levels and the efficiency of low-density lipoprotein (LDL) uptake of cells (Bartz et al., 2009). In this study knockdown of 100 pre-selected candidate genes was assayed for effect on cellular cholesterol homeostasis with two fluorescence-based assays in HeLa cells. The first assay was used to measure cellular cholesterol levels using a fluorescent cholesterol-binding dye Filipin. The second assay measured the dynamics by which LDL is internalized into cells. High-content microscopy and quantitative image analysis software were used to image the arrays and quantify the level of cytoplasmic Filipin and LDL against immunofluorescence counterstaining for endoplasmic reticulum or lysosomal marker LAMP1.

The fourth report using the same array technique described an analysis of genes involved in mammalian cell cycle progression with image based cytometry analysis of living cells (Neumann et al., 2010). HeLa cells with a GFP reporter expressed in fusion with a chromatin core histone H2B were transfected on hundreds of array slides with 384 siRNA spots on each and imaged with timelapse microscopy for two days. The image data and the resulting nuclear phenotypes of cells were scored quantitatively by a computational image processing allowing identification of altered cell cycle kinetics induced by the

RNAi gene silencing (Neumann et al., 2006). This study demonstrates that in addition to various immunofluorescence assay techniques, the cell microarrays provide also a screening platform compatible for performing high-throughput RNAi screening with live cells (Table 1.).

Table 1. Summary of different cell biological assays performed using high-throughput RNAi screening by transfection cell microarrays.

Reference	Assay type	Reporter	Cell Type(s)	Target genes
Silva et al., 2004	Epifluorescence analysis of proteasome functions.	α-tubulin GFP fusion construct.	HEK293T, HeLa, NIH3T3	30
Simpson et al., 2007	Epifluorescence and immunofluorescence analysis of secretory machinery.	CFP-coupled viral membrane protein. Antibody for tsO45G.	HeLa	37
Doil et al., 2009	Immunofluorescence analysis of P53BP1 recruitment to DNA	Antibody for P53BP1	U-2-OS	21,541
Bartz et al., 2009	Epifluorescence analysis of cellular cholesterol levels.	Fluorescent Filipin dye	HeLa	100
Neumann et al., 2010	Timelapse epifluorescence analysis of live cells	Histone H2B coupled with GFP reporter	HeLa	21,541

In conclusion, these studies provide examples demonstrating that cell microarray techniques can be successfully used for a variety of customized large-scale RNAi studies if certain limitations are solved. Robust reproducible production of the arrays and the automated microscopy used for analysis are the key aspects of performing and expanding cell microarray experiments for real high-throughput applications. A cell microarray technique with broad functionality is sufficiently flexible to not only allow proof-of-concept assays in a form of single primary type of screens, but also in additional cell types and potentially more complex screen contexts. In addition, the statistic scoring methods used for analysis of cell microarray screening data need to be carefully optimized to increase accuracy and reliability of cell microarray based high-throughput screening (Fjeldbo et al., 2008).

AIMS OF THE STUDY

Several groups have reported variations of the transfection cell microarray methods for high-throughput RNAi screening. Despite the multiple publications on the technique, the transfected cell microarrays have remained a difficult to adopt technology and hence failed to gain wide-spread use and redeem the great potential they hold. To enable robust reproducible array-format loss-of-function screening in a wide range of mammalian cell types and with multiple different assay types, we sought to develop a novel variation of the cell microarray technique to overcome some of the limitations of the previous methods.

The specific aims of this doctoral thesis were:

1. To establish conditions for production of cell microarrays with confined cell spots.

2. To validate functionality of the method in high-throughput screening applications.

3. To apply the method in disparate RNAi screening analyses.

MATERIALS AND METHODS

More detailed information on the methods is available in the original publications (I-IV)

Cell lines

Cell line name	Species	Tissue of origin	Used in
22Rv1	human	prostate adenocarcinoma	I, IV
HCC1937	human	ductal breast carcinoma	II
LAPC4	human	prostate adenocarcinoma	I, III
LNCaP	human	prostate adenocarcinoma	III
MCF7	human	breast adenocarcinoma	II
MCF-10A	human	breast epithelium	II
MDA-MB-231	human	breast adenocarcinoma	II
PC-3	human	prostate adenocarcinoma	I, IV
Primary stromal cells	human	prostate stroma	I
RWPE1	human	prostate epithelium	I
SVpgC2a	human	oral epithelium	I
T-47D	human	ductal breast carcinoma	II
U-2-OS	human	osteosarcoma	I
VCaP	human	prostate vertebral metastasis	I, III

Reagents and chemicals

Compound	Supplier	Used in
siRNA oligonucleotide libraries	(Qiagen)	I-IV
SiLentFect	(Bio-Rad Laboratories)	I-IV
Sucrose	(Sigma)	I-IV
GFR Matrigel	(BD Biosciences)	I-IV
OptiMEM I + GlutaMAX I	(GIBCO)	I-IV
4’,6-diamidino-2-phenylindole	(Invitrogen)	I-IV
Hoechst dye solution 33342	(Sigma)	I-IV
ProLongGold	(Invitrogen)	I-IV
Paraformaldehyde (PFA)	(Sigma)	I-IV
BSA	(Sigma-Aldrich)	I-IV
Triton X-100 solution	(Sigma-Aldrich)	I-IV
CellTiterBlue	(Promega)	I-IV
EdU	(Invitrogen)	II
Alexa Fluor phalloidin	(Invitrogen)	I-IV
Methyltrienolone (R1881)	(Perkin-Elmer)	III-IV
Bicalutamide	(Sigma-Aldrich)	III
Fibronectin	(Calbiochem)	I-IV
Collagen	(Inamed Biomaterials)	IV

Equipment

Type	Supplier	Used in
384-well low volume plates	(Abgene)	I-IV
96-well standard cell culture plates	(Corning)	I-IV
384-well standard plates	(Corning)	I-IV
Automated liquid handling robot	(Hamilton)	I-IV
Automated liquid dispenser	(ThermoFisher)	I-IV
QArray2 contact printer	(Genetix)	I-IV
Solid tip pins	(Point Technologies)	I-IV
Rectangular 4-well plates	(Nalge Nunc)	I-IV
Scanning microscope scanˆR	(Olympus Biosystems)	I-IV
LS400 Laser microarray scanner	(Tecan)	I-IV
BD FACSarray Flow cytometer	(BD)	IV
Accuri C6 Flow cytometer	(Accuri)	IV
Zeiss LS710 spinning disc confocal microscope	(Zeiss)	II-IV

Antibodies

Antigen	Species	Antibody	Used in
AR	mouse	Santa Cruz Biotechnology H-280	III, IV
β-tubulin	mouse	Santa Cruz Biotechnology G-8	I-IV
CAPN2	rabbit	Abcam ab713-50	I, IV
CD9	rabbit	Abcam ab92726	II, IV
CLDN4	goat	Santa Cruz Biotechnology N-20	IV
Cleaved PARP	mouse	Cell Signaling Technology 9546S	I-III
gamma H2A.X	rabbit	Abcam Ab2893	II
GINS2	chicken	Sigma-Aldrich GW22115F	II
ITGA1	rabbit	Millipore 1934	IV
ITGA2	rabbit	Millipore 1934	IV
ITGA2	mouse	Serotec MCA2025	IV
ITGA5	rabbit	Millipore 1949	I, IV
ITGA5	mouse	Chemicon 1999	IV
ITGB1	mouse	Abcam 12G10	I, IV
Ki-67	rabbit	Abcam Ab15580	I-III
PAK3	rabbit	Abcam EP797Y	IV
PTP4A3	rabbitg	Abcam ab50276-100	IV
ZO-1	mouse	Invitrogen 33-9100	IV

Methodology

Method	Used in
Cell culture	I-IV
Reverse transfection	I-IV
Western blot analysis	I-IV
Immunofluorescence	I-IV
Statistical analysis	I-IV
Proliferation assay	I-IV
Live cell microscopy	I, II, IV
Immunoprecipitation	III, IV
RNA extraction	I-III
Reverse transcriptase-PCR	I-III
Bioinformatics	I-IV
Flow cytometry	IV
Image cytometry	I-IV
In situ proximity ligation assay	III, IV
Gene expression analysis	II
aCGH	II
Adhesion assay	IV
Cell spreading assay	IV
Microarray scanning	I-IV
Microarray printing	I-IV

RESULTS

1. Cell spot microarray method (I)

In the vast majority of different cell microarray techniques, a sub-confluent layer of cells is seeded over the whole array surface covering both the printed samples and areas in between the spots. This feature of the so called 'cell carpet' approach is limiting to the accuracy of quantification of the transfected cells without the use of labelled siRNA reagents or co-transfected reporter gene constructs, as non-affected cells from the surroundings are able to intrude to the spot perimeter and mix with the transfected cells. For the development of an alternative technique to prepare cell arrays we sought to develop a technique which would allow preparing cell arrays with spatially confined cell spots to overcome limitations of the cell carpet arrays. To achieve this goal and develop a cell microarray format compatible with majority of adherent cell types, various array surface materials and combinations of different extracellular matrix (ECM) protein coatings, cell dissociation procedures and adhesion parameters for different cell types were cross tested. In the protocol resulting in the most efficient selective cell adherence to the printed matrix substrate in comparison to the array background, Matrigel ECM matrix is microarrayed with contact printing to a hydrophobic polystyrene surface (I: Fig.1A). Adherent cells dissociated with a collagenase based cell dissociation solution are dispersed over the array and allowed to adhere for a short period, commonly 5 to 15 minutes before unadhered cells, unable to make permanent contact onto the array background during the initial adherence incubation, are washed off. After the wash step cells are left growing only on the spatially confined spots, providing a platform compatible for ultra-high sample densities and simplified imaging and quantification of cells on the spots (I: Fig.1A & B). This approach was found to facilitate spatially restricted adhesion of the majority of the tested cell types (92%, 85/92) (I: Fig.2B). Due to the high precision of contact microarray printing the method can be scaled for ultra-high sample densities. With 200µm array spots and 500µm spot spacing, arrays with 3,888 spots in an area of 18x54mm or 15,552 spots in a single microplate-sized vessel with four large rectangular wells could be printed (I: Fig.2A). As the patterning of adhesion promoting matrix samples onto the arrays is done with contact printing the diameter of array spots and hence number of cells per spot can be controlled with printing pin diameter. Number of cells per spot is though dependent on the characteristics and growth properties of the cells. With epithelial derived PC-3 prostate cancer cells, number of cells on 200µm spots following 48h culture was found to be 51±3 (s.d., replicate spots n=100) and 151±8 on 400µm spots. In comparison, culture of primary un-immortalized prostate stromal cells on 200µm spots resulted in 21±6 cells per spot (I: Fig.1C).

To establish lipid based siRNA reverse transfection on the cell spot microarrays (CSMA), we aimed for low siRNA and lipid concentrations in order to suppress potential lipid toxicity and off-target effects. Efficacy of siRNA transfection on CSMAs was tested by printing 200µm array spots from 10-30ng/µl siRNA samples. Efficacy of exogenous target silencing was evaluated with U-2-OS sarcoma cells expressing a destabilized TurboGFP (TuGFP). Cells were transfected for 72 h on an array with 25 print replicates of a validated TuGFP siRNA in three concentrations (I: Fig.3A). TuGFP signal intensity was analyzed using automated microscopic analysis. With normalization of the cytoplasmic TuGFP signal to the nuclear DNA counterstaining (I: Fig.3B) of the cells, an up to 60% efficacy was measured with the lowest tested siRNA concentration, an over 70% efficiency was achieved with 20ng/µl siRNA samples and an up to 90% silencing efficacy was achieved with the 30ng/µl siRNA samples. To evaluate specificity of the siRNA induced effects with an endogenous target, transfection of a validated siRNA for CDC2 (*CDK1*, cyclin-dependent kinase 1) was used for the analysis (I: Fig.3A). With automated image cytometry analysis of the siCDC2 transfected cells, the CDC2 inhibition was found to induce a prominent G_2 cell cycle arrest with 51.1% of cells in G_2 after 72 h transfection with already the lowest siRNA concentration and an identical result with the higher siRNA concentration (20-30ng/µl) (I: Fig.3C). Control transfections and transfection with the TuGFP siRNA had no effect on the cell cycle distribution with all the siRNA concentration (I: Fig.3C). Silencing of an endogenous target gene was further validated with antibody based immunofluorescence detection of integrin alpha-5 (ITGA5) depletion in PC-3 prostate cancer cells. Here, an up to 75% average silencing efficacy was measured with 30ng/µl siRNA (I: Fig.3D). Endogenous target silencing was evaluated also with antibody based detection of Calpain-2 (CAPN2) silencing on VCaP prostate cancer cells transfected for 72 h on a CSMA with 96 technical replicates of four different *CAPN2* and control siRNAs (I: Fig.S1B). A mean transfection efficacy of 31 to 94% silencing per siRNA construct was achieved in comparison to the negative control siRNA transfected cells, using 10ng/µl siRNA concentration. To further validate the transfection efficacy with VCaP cells, a Western blot analysis of cells transfected for 72 h on an array with 384 (200µm) replicate spots of the most effective CAPN2 siRNA and an identical array of negative control siRNA was performed. Here, an up to 85% silencing efficacy was achieved, verifying the high transfection efficacy detected with the immunofluorescence. In comparison, an identical assay using PC-3 prostate cancer cells and SVpgC2a immortalized primary oral keratinocyte cells indicated up to 90 and 65% transfection efficiencies (I: Fig.S1D). This demonstrates the functionality of siRNA reverse transfection on CSMAs with both established model cell lines and primary cells.

2. Immunofluorescence based screening using the cell spot microarrays (I)

With the initial description of many of the transfection microarray methods, immunofluorescence assays have been tested, yet little or no data have been presented on the reproducibility of the assays in large-scale cell microarrays analyses. To validate the compatibility and reproducibility of the CSMA method for immunofluorescence based high content RNAi screening, we established an assay for cell proliferation and apoptosis using antibodies against Ki-67 and cleaved PARP (poly (ADP-ribose). With microscopic detection, the Ki-67 proliferation marker and cleaved PARP apoptosis marker show a mutually exclusive staining pattern allowing simple phenotypic delineation of proliferating and apoptotic cells on the basis of the nuclear staining intensity of the proteins against DNA counterstaining of the cells (I: Fig.4A & 4B). This assay was applied for analysis of G-protein coupled receptor (GPCR) coding genes impacting on the growth and survival of cultured prostate cells. A siRNA library with two siRNA constructs against 492 human GPCRs and replicate negative control siRNA samples was used for preparation of arrays used for parallel analysis of GPCRs important for growth of androgen responsive VCaP and LAPC-4 prostate cancer cells and RWPE-1 non-malignant prostate epithelial cells to evaluate accuracy of the CSMA based screening in different cell types.

For the screens, two identical arrays with individual siRNA samples printed in a random order were used to transfect each cell type for 48 h followed with immunofluorescent staining of the cells for analysis using automated microscopic imaging. Each array position was imaged and subsequently analyzed using automated fluorescence microscopy and image analysis software. In the image analysis, cells were segmented on basis of the DNA staining and the nuclear staining intensities of the markers were quantified (I: Fig.4B). The spot level ratio of RNAi induced changes in the cumulative nuclear intensities of Ki-67 and cPARP were used to identify target genes causing inhibition of cell proliferation and induction of apoptosis upon silencing. By comparison of the scored results of the two replicate arrays for each cell type, the CSMA experiments displayed a statistically significant concordance with Pearson correlations between r=0.79 to 0.84 (I: Fig.4C & 4D) demonstrating reproducibility of the CSMA screening in different cell types. With comparative analysis of the CSMA screening results, *NPY* (Neuropeptide Y) was identified as the strongest common growth inhibitory siRNA hit in all the three cell lines (I: Fig.5B), while *GPR160* (G protein-coupled receptor 160) had the strongest growth inhibitory effect on the cancer cell lines VCaP and LAPC-4, and an insignificant effect on the RWPE-1 epithelial cells (I: Fig.5B-5C). Bioinformatic analysis of published gene expression studies of clinical prostate cancer samples was used to evaluate clinical significance of these genes. Here, both NPY and GPR160 were identified to display an increased expression pattern in the prostate cancer samples (I: Fig.5A). The

growth inhibitory knockdown effect of NPY and GPR160 depletion identified in the immunofluorescence assay on CSMAs was validated in a series of secondary validation experiments verifying accuracy of the original screens. Results from the screens indicate that the CSMA method allows both reproducible immunofluorescence based screening as well as accurate analysis of multiple cell types. The screens also highlighted two GPCR coding genes as potentially the most tissue specific GPCRs important for prostate cancer cell growth potentially also *in vivo*.

3. Cell morphology based screening (II)

To establish conditions for using the cell spot microarray method for screening of large-scale siRNA libraries and cell morphology based assays, a study for analysis of genes inducing polyploidy upon depletion in aneuploid breast cancer cells was performed. Aneuploidy is among the most obvious differences between normal and cancer cells. However, mechanisms contributing to development and maintenance of aneuploid cell growth are diverse and incompletely understood. Previous functional genomics analyses have shown that aneuploidy in cancer cells is correlated with diffuse gene expression signatures. Aneuploidy can arise by a variety of mechanisms, including cytokinesis failures, DNA endoreplication and possibly through polyploid intermediate states. To identify molecular processes contributing to development or maintenance of aneuploidy, we performed a cell spot microarray RNAi analysis to identify genes inducing polyploidy and/or allowing polyploid cell growth in breast cancer cells. Arrays with two siRNA for 5,760 human genes were used to transfect MDA-MB-231 cells for quantification of polyploid cells. In the conventional RNAi screens, cells are commonly exposed to the RNAi for 48 to 72 hours, after which the cells are prepared for analysis. This allows identification of the immediate and initial phenotypes resulting from the gene silencing, but the long term impact of the gene silencing is not considered. In the performed screen, the cells were exposed to the siRNA for seven days to highlight especially cell cycle defects resulting in formation of severe sustained polyploidy (II: Fig.1A). Cells were seeded onto the CSMA and prepared for microscopic imaging after seven days (II: Fig.1C). Polyploid cells were quantified on the basis of nuclear size alteration and compared to automated quantification of total cell number on the array spots to determine an index of polyploid cells induced by the target gene silencing. The identified polyploidy phenotypes were further classified as sustained or apoptotic polyploidy on basis of the nuclear morphology (II: Fig.2A). Based on the prevailing phenotype, the candidate genes inducing a greater than 20% penetrance of polyploid cells per spot were divided into these two categories (II: Fig.2A). With the threshold of 20% polyploid cells per spot and two independent analyses of the resulting phenotypes, silencing of 177 genes were considered positive (3% hit rate) with 28 genes scoring with

both of the used siRNAs. Based on the phenotypic stratification, 134 of the candidate genes were classified to induce sustained polyploidy and 43 apoptotic polyploidy upon depletion.

Bioinformatic functional annotation of the identified candidate genes was used to address which biological processes are enriched within the highlighted target genes. Association with response to DNA damage stimulus and DNA repair were found to be the most enriched cellular processes among the candidate genes (II: Fig.2C). Secondary CSMA screening of these genes with immunofluorescence assays for cell proliferation, induction of DNA damage and apoptosis highlighted GINS2 (GINS complex subunit 2) as the highest ranking candidate inducing polyploidy, accumulation of endogenous DNA damage and impairing cell proliferation upon inhibition (II: Fig.3B). The cell growth inhibition and induction of polyploidy by suppression of GINS2 was verified in a panel of aneuploid breast cancer cell lines (II: Fig.5A). Bioinformatic analysis of published gene expression and DNA copy number studies of clinical breast tumors suggested GINS2 to be associated with the aggressive characteristics of a subgroup of breast cancers *in vivo* (II:Fig.4 & Fig.5D). In addition, nuclear GINS2 protein levels distinguished actively proliferating cancer cells suggesting potential use of GINS2 staining as a biomarker of cell proliferation as well as a potential therapeutic target (II: Fig.5C). These results indicate that the CSMA method can be used to prepare cell microarrays with up to systemic scale RNAi reagent coverage in a single array plate. In this study the CSMA comprised 15,552 sample spot printed on a single CSMA plate. Moreover, the study demonstrates that the cell spot microarray method allows in addition to the immunofluorescence based assays also cellular phenotype based siRNA screening as well as innovative assay such as the prolonged RNAi used for the analysis.

4. Combinatorial RNAi analyses using cell spot microarrays (III)

The functional effect and cell biological importance of different genes and gene products occurs as a function of tissue type. Different signaling networks and individual genes function in a highly delicate equilibrium in cells of different tissues of origin. Regulation of this context dependent balance is implicated in the development of eukaryotic organisms through epigenetic and transcriptional regulation of different genes in different tissues and phases of development. Cancer is also a heterogeneous disease that arises from a multitude of genetic and epigenetic alterations. The loss of function of a tumour-suppressor gene and/or the gain of function of a dominant oncogene may disrupt the normal balance of different signaling networks and therefore drive cancerous cell growth. Deregulation of specific oncogenes and tumour suppressor genes occurs also as a function of tissue type and environmental conditions, as some genes have dual

functions depending on the type of cancer. The same gene can have tumour suppressor-like activities in one type of cancer, but function as an oncogene in a different cancer context, as for example TGFB1 (transforming growth factor-β) (Siegel & Massague 2003) and CDKN1A (cyclin-dependent kinase inhibitor 1A) (Roninson 2002). Hence, systematic context-specific analysis of gene-environmental gene-gene or gene-drug interactions is critical towards an improved understanding of cellular and cancer biology, as well as pharmacogenomics.

RNAi screening provides unique opportunities for combinatorial analysis of gene functions. However, due to the enormous scale of possible combinations involved, these interactions are often not experimentally testable using multiwell based screening techniques. Moreover, the cell microarray methods where such comprehensive gene perturbations would be more feasible to perform have not been tested in these types of experiments. Here, we demonstrate the utility of the cell spot microarray method for combinatorial RNAi analyses with a screen of genes sensitizing prostate cancer cells to androgen deprivation induced growth inhibition. The androgen receptor plays a pivotal role in the development and progression of prostate cancer and represents a key target for drugs to inhibit the effects of androgen signalling in prostate cancer. Since androgen-deprivation therapy alone is not curative, we sought to identify genes that sustain the growth and survival of prostate cancer cells in androgen-deprived conditions. We performed a comparative RNAi screening of 2,068 genes, including kinases, phosphatases and epigenetic enzymes, both in the presence and absence of androgens using cell spot microarrays. As the model we chose to focus on VCaP cells, which are androgen responsive, harbour the genomic amplification of the *AR* and express the TMPRSS2-ERG fusion transcript (Korenchuk et al., 2001). With CSMA reverse transfections, VCaP cells were verified to display high transfection efficacy and assay reproducibility (I: Fig.S1B). For the screens, VCaP cells were first transfected in normal growth medium for 24 h on two identical arrays, after which one of the arrays was changed to androgen deprived growth medium. The other one was kept in the normal growth medium for an additional 48 h. After a total of 72 h transfection and culture, cells were fixed and stained with antibodies against the Ki-67 proliferation marker and cleaved PARP apoptosis marker for microscopic detection of proliferating and apoptotic cells (I: Fig.4A & B, III: Fig.1B). The resulting comparative analysis of over 24,000 siRNA/phenotype correlations under these two conditions (III: Fig.1C, 2A) led to the identification of 35 genes with a growth inhibitory effect specifically in the androgen deprived conditions as measured by the Ki67 to cPARP signal ratios. With analysis of the two antibody markers independently; siRNAs targeting 122 genes resulted in increased cPARP staining and 27 genes scored specifically on decreased Ki-67 index in response to androgen deprivation (III: Fig.2B). Bioinformatic analysis of the identified genes across major human tissue and tumor types indicated that a substantial fraction of the highest

ranking candidate genes were highly expressed in clinical prostate samples *in vivo* (III: Fig.S1). One of such candidates, a transcriptional activator HSPBAP1, was identified to display increased expression levels in 43% of localized prostate cancers (III: Fig.3A). In molecular biological validation experiments, HSBPAP1 was validated as a protein whose interaction with the androgen receptor in the prostate cancer cell nuclei was activated by androgen-deprived conditions, facilitating the survival of prostate cancer cells in androgen-deprived conditions (III: Fig.3D & E). Furthermore, HSPBAP1 was identified to be an androgen receptor target gene (III: Fig.4B) and that depletion of HSPBAP1 transcription attenuated also AR transcription (III: Fig.4A & D).

In summary, this study demonstrates that CSMA analysis facilitates combinatorial strategies for large-scale gene knockdowns coupled with environmental challenges. Moreover, our data suggest a novel role and a possible link for HSPBAP1 in promoting prostate cancer cell survival in androgen-deficient conditions. These results also highlight the critical importance of better understanding context-specific gene and protein interactions for the development of novel therapeutic interventions in prostate cancer.

5. Next generation high-throughput applications (IV)

Due to the high reagent demand of conventional multiwell based high-throughput screening platforms, the majority of described RNAi screens have relied on the exploration of RNAi knockdown phenotypes on basis of the analysis of a single assay parameter, such as cell morphology, reporter gene assay or immunofluorescence marker at a time. Also the previous cell microarray RNAi studies were performed with epifluorescence analysis of a single reporter gene or single immunofluorescence readout at a time. Though well suited for specific cell biological questions, these approaches ignore the overall complexity of the molecular biology. Moreover, many informative assay types are not applicable to high-throughput screening due to the limitations arising from the multiwell platforms. Based on the miniature nature of the cell microarray methods, several alternative assay techniques could be used for functional genomics screening with these platforms. For example antibody staining of cell microarrays can be performed using a coverslip to cover the whole array surface, thus significantly reducing the volume of antibody solutions required for staining of highly parallel transfected cell microarrays. To demonstrate the use of cell spot microarrays for next-generation type functional genomics screening, we performed an analysis of the regulation of endogenous protein-protein interactions with proximity ligation assay detected *in situ* analysis of interacting integrin protein pairs.

To a great degree, all cellular processes depend on serial co-operation of pairs of proteins and coordinated interaction of molecular complexes. Hence, analysis of protein-protein interactions is essential for the elucidation of protein complexes and signaling networks involved in the regulation and proper functioning of cellular processes. Analysis of endogenous protein interactions offers also profound insights into the alterations of normal processes involved in pathogenesis of disease states, including cancer. Although several methods exist to study endogenous protein interactions in isolation, large-scale functional analysis of the regulation of complex formation and spatial localization of *in situ* protein interactions in cells remains a difficult endeavour. To develop a new highly parallel approach for functional genomics analysis of endogenous interacting proteins, we applied RNAi screening using cell microarrays to explore and visualize the regulation of endogenous protein interactions with antibody-based proximity ligation assay (PLA). In the PLA method, interacting target proteins are detected in fixed cell samples using paired primary antibodies followed with probe antibody recognition for formation of a PLA detection complex (IV: Fig.1A). Each detection complex is used as a template for localized rolling circle amplification reaction, generating a long single-stranded DNA molecule, which can be labeled with fluorescent DNA probe molecules. The resulting bright fluorescent PLA complexes can be imaged by standard fluorescence microscopy for parallel quantification and spatial definition of interacting protein pairs (IV: Fig.S1A). Previously PLA has been used to study protein-protein interactions and protein co-localizations in context of individual biological experiments. Recently PLA technique was used also in a HTS application, but the reagent intensive detection method is limiting to the assay scale of microplate format applications. With CSMAs, a detection reaction equivalent for PLA staining of a single 96-microplate well is sufficient for staining an array with up to 768 different siRNA transfections, thus significantly reducing reagent consumption.

As the proof-of-concept functional genomics PLA analysis, we performed a comparative siRNA screening of genes impacting on the activation of heterodimeric beta-1 integrin (ITGB1) cell adhesion receptors. For the experiment, 125 genes highlighted with a potential role on regulation of ITGB1 ligand affinity in an ongoing CSMA RNAi screen were selected for parallel loss-of-function profiling of their specific impact on the collagen binding heterodimeric integrins α1β1 and α2β1 and fibronectin receptor integrin α5β1 activation (IV: Fig.1B). With automated microscopic analysis of the PLA assays (IV: Fig.2A), we demonstrate that the activation response of the collagen receptors share a high degree of concordance, whereas the fibronectin receptor integrin α5β1 was found to be selectively affected also through an alternative network of functionally associating molecules (IV: Fig.2C & 3A). A set of secondary validation assays including flow cytometric analysis of integrin ligand binding were used to validate the CSMA screening results (IV: Fig.4A-D), indicating a strong correlation between the CSMA screening

results and the results of transfection of the same siRNAs in conventional multiwell plates for integrin activity analysis.

With comparison of the biological results of the PLA screening, CLDN4 (Claudin-4) depletion was identified among the candidates with the strongest integrin α5β1 specific activity reducing phenotype, indicating that CLDN4 may play a specific role in enhancing the formation of active integrin α5β1 complexes (IV: Fig.5A-F). CLDN4 has been shown to be frequently over-expressed in clinical prostate tumors and several other epithelial cancer types and the dysregulation of CLDN4 expression is associated with the epithelial to mesenchymal transition (EMT) and metastatic development in clinical prostate cancers. In molecular biological validation experiments, we verified that CLDN4 interacts with ITGA5 and that this interaction enhances the activity of ITGA5 complexes (IV: Fig.5A & C) supporting integrin α5β1 mediated functions, such as cell adhesion to fibronectin and motility on plastic. In prostate cancer cells, we verified CLDN4 to be under androgen receptor regulation (IV: Fig.6B-D), making the discovery of high contextual importance.

Taken together, these results demonstrate the use of cell spot microarray for high-throughput RNAi analysis with assay techniques otherwise difficult or not applicable to functional genomics screening. Moreover, the analysis demonstrate the accuracy of PLA based screening of *in situ* protein interactions with the first described comparative analysis of molecules impacting on the activity of different heterodimeric integrins.

DISCUSSION

1. CSMA method

Here, we describe the development, large-scale application and validation of the methods for production and analysis of the cell spot microarrays. As compared to other cell microarray methods, where a carpet of cells covers the entire array, CSMA provides a patterned layout, with the cells only adhering to the spots containing the siRNA-lipid samples and an ECM coating. This significantly facilitates digital image analysis and essentially enables the analysis of higher sample densities. Using the described array settings, up to genomic-scale siRNA collections can be printed and analyzed using a single array plate. Furthermore, we report combination of the CSMA application with multiplexed high-content antibody-based assays, using a wide range of different cell types, live cell imaging, in combinatorial RNAi analysis and with assay techniques otherwise not applicable to high-throughput screening thereby greatly expanding the scope of the transfected cell microarray applications. Based on our approach, only cell dissociation protocols and adherence times required optimization, otherwise an identical protocol was used for array printing, composition of the siRNA spots and all cell lines.

The consumption of siRNAs required for individual transfection on CSMAs was up to 200-fold less compared to traditional plate-based screening in a 384-well format (10ng/well vs. 50pg/spot). Additionally, immunofluorescence staining of CSMAs reduced antibody consumption up to 400-fold as compared to immunostaining in 384-well format (10µl/well vs. 100µl/array area with 3840 spots). The addition of ECM matrix proteins was demonstrated to enhance the adherence and growth of a multitude of cell types on the CSMA spots. This makes it possible to adapt rare and slowly growing cell types, such as primary epithelial or tumor cell cultures for CSMA analysis. As extra cellular matrix proteins have been demonstrated to provide a more physiological cell culture environment in comparison to plastic cell culture surfaces, it is also intriguing to speculate that the CSMAs could provide a more physiological platform to study gene functions in comparison to plastic multiwell plates cell microarrays prepared on plain glass.

2. Biological applications

The first biological application utilized here to demonstrate the functionality of the CSMA method in high-throughput screening was the analysis of G-protein coupled receptors (GPCR) important for growth and survival of cultured prostate cancer cells.

This work provided grounds for the systematic screening of siRNA libraries using immunofluorescence methods combined with automated microscopic imaging on cell spot microarrays. The analysis is the first described HTS study where multiple antibody markers are used in parallel for RNAi analysis. The study is also the first described RNAi screening where primary analysis of knockdown phenotypes is performed in parallel in different cell types. Here, two cancerous cell lines were screened in parallel to non-malignant prostate epithelium cells demonstrating the compatibility of the method for use with different human cell types. Moreover, the study is the first describing functional analysis of GPCR coding genes in prostate cancer cells and identifying candidate GPCRs potentially important for prostate cancer cell growth also *in vivo*. The second biological application described use of the CSMA method for cell phenotype functional genomics screening. Here a siRNA library for 5,760 human genes and arrays with 15,552 sample spots was used for the analysis, demonstrating 1: The production of highly parallel CSMAs, and 2: Application of CSMA for phenotype based RNAi screening. The performed study was aimed on analysis of genes inducing severe polyploidy upon depletion. The analysis identified a relative poorly studied candidate gene GINS2, which was found to display significant potential for use as a molecular marker for delineation of aggressive breast cancers *in vivo*. The study is the first described RNAi screening where transient gene silencing is applied for a pro-longed period to highlight cell phenotypes arising from the sustained gene knockdowns. This feature of the CSMA method is of particular importance for future RNAi screening studies intended for discovery of novel therapeutic targets, as genes with accumulating depletion effects could prove to be valuable target molecules in e.g. cancer treatment.

The third application described analysis of target genes that sensitize androgen-sensitive VCaP prostate cancer cells to androgen deprivation. In this screen, we demonstrate use of the CSMA method for combinatorial RNAi analysis which has not been previously described with cell microarray methods. The study identified a group of candidate genes that were shown to be functionally relevant for prostate cancer cell survival exclusively under androgen-reduced growth conditions. A subsequent bioinformatic analysis of the hit genes identified in the screening further narrowed down a group of targets that were likely to be of the highest clinical relevance. This group included several known AR-interacting genes, co-activators or candidates with a previously established association with prostate cancer and androgen independent growth, such as ATAD2, IRS2, JMJD2B, SVIL, PTGS2, NOV and SRD5A2, indicating the accuracy of the CSMA method for context specific target discovery. Furthermore, genes without any previous connection to prostate cancer or androgen receptor signaling were also identified. Our data therefore verifies the compatibility of the CSMA method in combinatorial RNAi analyses using multiplexed immunofluorescence assays, and highlight potential therapeutic

opportunities arising from the studies of context-specific gene-condition interactions using the CSMA method.

The fourth application example demonstrated use of CSMAs with next generation assay techniques. Here, proximity ligation assays for analysis of protein interactions *in situ* were applied to evaluate accuracy of CSMA mediated analysis of functional regulation of endogenous protein interactions. A comparative RNAi analysis of active state integrin complexes was performed validating the compatibility of CSMAs as a platform for reagent demanding assay methods not previously described in functional genomics analyses. Possibility to analyze endogenous protein interactions in high-throughput mode is an invaluable tool for future molecular cell biology exploration. Even though many methods exist to study protein interaction in cells, these techniques are often limited to single assays not scalable for thousands of samples and moreover, they rely on exogenous expression constructs lacking potentially the endogenous post-translational modifications of the proteins and natural transcriptional regulatory mechanisms. The advantages of the described approach are therefore many folded in comparison to conventional fixed endpoint immunoassays. From the biological point of view, the study describes also the first systematic attempt to identify molecules with specific roles on functionality of different heterodimer integrins. Resulting from the analysis, CLDN4 was identified as a novel molecule specifically enhancing activity of integrin α5β1. CLDN4 was identified to associate with ITGA5 and furthermore we demonstrated that in prostate cancer cells transcription of CLDN4 is under regulation by androgen receptor providing evidence of a novel mechanism contributing to the overexpression of CLDN4 in clinical prostate tumors.

3. Transfection cell microarray techniques in future

The great potential of RNAi-mediated loss-of-function genetics has altered the philosophy of biological exploration of gene functions. The continuous development of RNAi technology will in future further expedite the more comprehensive delineation of gene functions in normal physiology and disease states.

Transfection cell microarrays offer a unique platform for carrying out highly parallel loss-of-function studies. As the siRNA (shRNA) libraries become more complete and validated and the cell microarray technology progresses, cell arrays for analysis of genome-wide libraries of RNAi reagents will allow inexpensive, fast and accurate functional characterization of human genome in days instead of weeks or months. The most important advantages of the cell spot microarray method for future RNAi applications are the high sample density allowed by the technique and independency

from extensive infrastructure that makes functional screening possible without the expensive screening facilities. When the cell spot microarrays become more widely available, research laboratories would only need compatible imaging instrumentation to conduct rapid, customized large-scale RNAi screens. Furthermore, the cell spot microarray formats will allow the use of more sophisticated assay methods and rare cell types such as primary cultures for functional genomics screening.

SUMMARY AND CONCLUSIONS

A cell microarray platform with spatially confined cell spots was developed. Synthetic short interfering RNA (siRNA) libraries targeting human genes were used to establish high-throughput RNAi screening with the platform. To test the biological utility of the technique, we applied it to a set of independent siRNA-experiments that focused on the identification of target genes required for the growth and survival of human prostate cancer cells, maintenance of genomic integrity in aneuploid breast cancer cells, and in the activation of integrin complexes. The screens demonstrated the compatibility of the method with various different assay techniques and identified several novel candidate regulators of cancer cell biological processes. Bioinformatic and cell biological assays of the identified genes were successfully used for validation of the hits identified using CSMA screening. This work therefore provides a protocol for a robust widely applicable and accurate cell microarray method for loss-of-function screens, as well as an exemplar for its application to cancer cell biological discovery.

Finally, although transfection cell microarrays as a whole are still an evolving technology, they hold the potential to significantly enhance the scope of functional exploration of genes in the future. As the CSMA technique matures, it will aid in the rapid systematic functional annotation of human genes. The cell spot microarray method offers also flexibility in carrying out high-throughput experiments with assay types otherwise not applicable in large-scale screening. Combinatorial experiments in search of e.g. synthetic phenotypes using cell spot microarrays as demonstrated here could also prove very effective in the search for novel therapeutic approaches by gene-drug interactions. In conclusion, the CSMA method makes it possible to design and complete sophisticated next-generation high-throughput and high-content assays not feasible or affordable with the conventional methods.

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