# High Throughput Screening of Gene Functions in Mammalian Cells Using Reversely Transfected Cell Arrays: Review And Protocol

Michael Stürzl[*,1], Andreas Konrad[1], Gaby Sander[1], Effi Wies[2], Frank Neipel[2], Elisabeth Naschberger[1], Simone Reipschläger[1], Nathalie Gonin-Laurent[1], Raymund E. Horch[3], Ulrich Kneser[3], Werner Hohenberger[4], Holger Erfle[5] and Mathias Thurau[1]

[1]*Division of Molecular and Experimental Surgery, Department of Surgery, University of Erlangen-Nuremberg, Schwabachanlage 10, D-91054 Erlangen, Germany*

[2]*Institute of Clinical and Molecular Virology, University of Erlangen-Nuremberg, Schlossgarten 4, D-91054 Erlangen, Germany*

[3]*Department of Plastic and Hand Surgery, University of Erlangen-Nuremberg, Krankenhausstrasse 12, D-91054 Erlangen, Germany*

[4]*Department of Surgery, University of Erlangen-Nuremberg, Krankenhausstrasse 12, D-91054 Erlangen, Germany*

[5]*BIOQUANT-Zentrum, Ruprecht-Karls-Universität Heidelberg, Im Neuenheimer Feld 267, D-69120 Heidelberg, Germany*

**Abstract:** Reversely transfected cell microarrays (RTCM) have been introduced as a method for parallel high throughput analysis of gene functions in mammalian cells. Hundreds to thousands of different recombinant DNA or RNA molecules can be transfected into different cell clusters at the same time on a single glass slide with this method. This allows either the simultaneous overexpression or - by using the recently developed RNA interference (RNAi) techniques - knockdown of a huge number of target genes. A growing number of sophisticated detection systems have been established to determine quantitatively the effects of the transfected molecules on the cell phenotype. Several different cell types have been successfully used for this procedure. This review summarizes the presently available knowledge on this technique and provides a laboratory protocol.

**Keywords:** Reverse transfection, cell microarray, cell chip, apoptosis, proliferation, siRNA.

## INTRODUCTION

In the year 2000, the complete sequence of the human genome has been announced. One of the major findings of this ambitious enterprise, which started 15 years ago, was that the human genome is composed out of around 30.000 genes [1-3]. This number sets a very important milestone in the understanding how biochemically manifested genetic information may be translated into the development of human beings, providing for the first time a complete picture of all the players involved.

However, the sequence of the genome alone gives only a static picture. It is well known that the development of organisms requires dynamic regulation of gene activity and interaction of different gene products in a carefully time and space coordinated manner. This involves the regulation of promoter activity, the processing of gene expression products at the RNA level (splicing), the regulation of translation, and the introduction of protein modifications, only to mention the most important regulatory checkpoints of gene activity. Considering that different gene products can cooperatively interact opens myriads of possibilities how gene activity can be regulated in development and diseases. This demonstrates that the understanding of the detailed molecular mechanisms how information statically encoded in the DNA gives rise to an organism is still at the very beginning.

Further technical improvements allowing the analysis of the dynamics of gene activity at the full genome level are required. Important progress has been made by the development of high throughput approaches which allow the analysis of the complete gene expression pattern of mammalian cells and tissues at the transcriptome level with array technology [4]. In addition, methods to determine the complete proteome of mammalian cells using high throughput mass spectrometry and protein microarrays [5] are constantly improving. New methods which provide a complete display of the major differences of the proteomes of different cell types or tissues are available, such as 2-D difference gel electrophoresis (DIGE) [6]. Finally, it is possible to analyze protein interactions with yeast and mammalian two-hybrid analysis and the tandem affinity purification (TAP)-tag method [7-11].

Until recently, the analysis of gene functions did not keep up with the aforementioned high throughput technologies to determine gene expression or protein-protein interactions. Available technology predominantly allowed analysis on a gene-by-gene scale. To this goal a DNA construct was expressed within cells either directing the overproduction of a gene product or inhibiting the expression of a gene of inter-

*Address correspondence to this author at the University of Erlangen-Nuremberg, Department of Surgery, Division of Molecular and Experimental Surgery, Schwabachanlage 10, D-91054 Erlangen, Germany; Tel: ++49-9131-85-33109; Fax: ++49-9131-85-32077;
E-mail: michael.stuerzl@uk-erlangen.de

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est. Subsequently, the effects of these manipulations were measured by different cell biological tests. It is clear that on a gene-by-gene basis the allocation of the functions of the 30.000 genes in mammalian cells will be a long lasting task and that it will be unworkably to determine combination effects of gene products on a systematic base and in different cell systems. Ongoing research programs aim to increase the speed of gene function analysis in mammalian cells by (1) miniaturization of assays, (2) automatization of experimental processes and (3) improvement and automatization of data recording and management. The goal is to carry out many different tests in parallel on a reduced cost basis for the single test. In most ongoing projects this is achieved by assembling fully automated laboratories where analyses are carried out by robots in a 96-well or 384-well format.

In 2001, Ziauddin and Sabatini succeeded in scaling down high throughput gene function analysis to the microarray level [12]. Different cDNA expression plasmids were spotted onto slides using a microarray roboter. The dried slides were exposed to a transfection reagent, placed in a culture dish and covered with adherent mammalian cells in medium. This created microarrays of simultaneously transfected cell clusters with different plasmids in distinct and defined areas of a lawn of cells. This method did not require the use of individual wells to separate the different plasmids or the clusters of differently transfected cells. The process of creating a microarray of clusters of transfected cells was called transfected cell microarray. The transfection method was named reverse transfection, because in contrast to conventional transfection protocols, DNA was "seeded" first and subsequently the cells were added. The reversely transfected cell clusters potentially can be screened for any property detectable on a surface or affecting its phenotype. The identity of the responsible cDNA is defined from the coordinates of the cell cluster on the reversely transfected cell microarray (RTCM).

In the mean time RTCM technology has been established by several different laboratories worldwide using different conditions, cell types, and approaches to detect variations in the cell phenotypes. In this review we will summarize the present knowledge related to this methodology.

Investigation of the function of a specific gene is carried out by either overexpression or silencing of the gene of interest. As yet, RTCM technology has been most commonly used to overexpress proteins in order to study protein localization, interaction of proteins with binding factors and the effects of the respective proteins on the cell phenotype. Using these approaches, particular care has to be taken because artifacts can be induced by expression of non-physiological high amounts of the gene product with strong heterologous promoters [13].

Gene silencing approaches can be used alternatively. An efficient methodology to perform this task is only available since recently and therefore is just at the beginning to be applied to RTCM technology [14]. In 2001, it was demonstrated that RNA interference (RNAi) can operate in mammalian cells and it was realized that RNAi represents a very convenient method for gene silencing approaches [15]. RNAi pathways are running through small RNAs which include micro (mi)RNAs and short interfering (si)RNAs (Fig. **1**). miRNAs are derived from non-coding hairpin RNA structures that are naturally transcribed from the genome whereas siRNAs are recombinatly produced [16, 17]. miRNAs mediate repression of translation and degradation of imperfectly complementary target transcripts. In contrast siRNA can induce gene silencing through sequence specific cleavage of full complementary messenger RNA (mRNA) only. Gene silencing by mRNA cleavage is particularly potent because the cleaved mRNA is rapidly degraded and the activated RNA-induced silencing complex (RISC) is released to react with and destroy another mRNA [18]. Synthetic siRNAs are either directly transfected into the cytoplasm or various vectors are used to express short hairpin (sh)RNA. Transfected siRNAs bypass earlier steps in the RNAi silencing pathway and are directly loaded into the RISC complex [19] (Fig. **1**). Experimental artifacts by siRNA usage may be due to unspecific effects on the expression of other genes but may also be envisioned by loading of the cells with foreign nucleic acids, as it has been observed with the use of antisense oligonucleotides. These were regarded as "magic bullets" for gene silencing approaches until significant side reactions like induction of the interferon system and shifts of the nucleotide pool by degradation of the introduced nucleic acids were discovered. Therefore, both methods, overexpression and silencing of genes, require careful controls especially when carried out on a genome-wide basis.

## RTCM TECHNOLOGY AND OVEREXPRESSION OF GENES

Overexpression of genes was carried out in several different studies using the RTCM technology for different purposes, with different cell types and different detection systems (Table **1**). In their original work Ziauddin and Sabatini showed that RTCM technology may be useful for the identification of drug targets, for the discovery of gene products which may alter cellular physiology, and for characterization of sub-cellular protein localization [12]. They expressed cDNA encoding immunophilin FKBP-12 (FK506 binding protein) in HEK293T cells. FKBP-12 is a binding partner of FK506 (also tacrolimus or fujimycin), a clinically important immuno-suppressant. Then they added radiolabeled FK506 to the tissue culture medium and showed by autoradiograpy that FK506 bound to the array in a pattern, which exactly matched the cell clusters expressing FKBP-12. In a similar approach they demonstrated binding of the antagonist SCH23390 to the D1 dopamine receptor. In addition, they expressed 192 V5-epitope-tagged cDNA molecules cloned in expression vectors. Using immunofluorescence they could identify cell clusters with increased levels of kinase signaling activity and altered cellular morphology. Additionally, TUNEL tests were used to screen the effects of the cDNAs on apoptotic signaling pathways.

***Transfection Technology:*** A major challenge for the broad applicability of the RTCM method was its potential usage with cell types different from HEK293T cells. Several studies have addressed this point.

Yoshikawa and colleagues described the transfection of primary human mesenchymal stem cells [20]. This required addition of basal membrane protein fibronectin to the transfection mix, which was spotted onto the slides. Another study confirmed that seeding of the cells on basal membrane

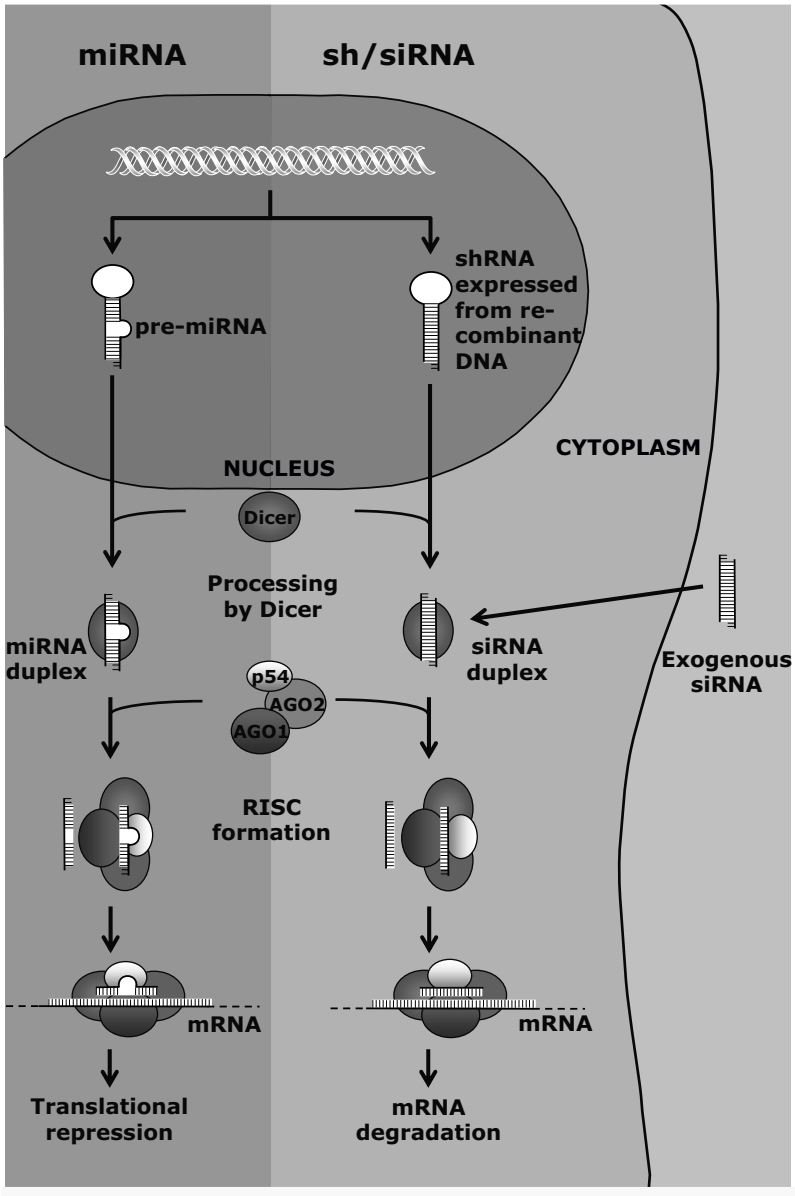


**Fig. (1). miRNA- and siRNA-induced gene silencing.** miRNA genes are transcribed by RNA polymerase II (left). RNA molecules, which form a hairpin structure with a partially complementary stem region and a 2 nt single-stranded overhang at the 3' end (pre-miRNA) are generated by RNA processing. Pre-miRNA is exported from the nucleus to the cytoplasm and is processed by the ribonuclease Dicer into a small miRNA duplex of approximately 20 nt in size. The miRNA duplex is incorporated into the RNA-induced silencing complex (RISC). This complex contains different proteins like Argonaut-1 and -2 (AGO1 and AGO2) and p54. The sense RNA strand of the miRNA duplex is released from the complex, which leads to activation of RISC. Activated RISC binds to appropriate mRNA molecules and inhibits translation. shRNA is usually ectopically expressed in cells by the usage of viral vectors (right). Transcribed shRNA consists of a hairpin loop and a fully complementary stem structure with a 2 nt single stranded protrusion at the 3' end. shRNA is exported into the cytoplasm and processed by Dicer into a small RNA of about 20 nt (siRNA duplex). Alternatively, siRNA duplexes can be introduced into the cell exogenously by transfection. siRNAs are incorporated into the RISC and after RISC activation by sense strand displacement the degradation of the respective complementary target mRNA molecules is conducted by RISC.

proteins can enhance the transfection efficiency of certain cell types in the RTCM method. For example transfection efficiency of pheochromocytoma cells could be significantly increased when the slides were coated with collagen IV [21]. It has been shown that those coatings which increased transfection efficiency simultaneously increased cell spreading and intracellular free calcium concentrations as detected with Fura-2 staining [20, 21]. The latter may activate actin filament reorganization, increase phagocytosis and endocytosis and *via* this may actively contribute to the uptake of DNA into the cell. Another study indicated that the effect of coating on transfection has to be carefully controlled and may sometimes be based on secondary effects [22]. Slides which had been coated with different substrates with decreasing

**Table 1. Different Approaches of High Throughput Screening of Gene Functions in Mammalian Cells by Reversely Transfected Cell Arrays**

| Goal | Cell Type | Reporter System | Coating | Transfection Reagent | Spot Diameter | Ref. |
|---|---|---|---|---|---|---|
| **Overexpression of proteins** | | | | | | |
| Identification of drug targets, cellular localization of proteins and their effects on cellular physiology | HEK293T | Binding of radiolabeled factors and autoradiography, p-Tyr, p-MAPK, Myc-, HA-, V5-tag IF | GAPS | Effectene (Qiagen) | 120-150 µm | [12] |
| Identificaton of genes activating MAPK and JNK pathways | HEK293 | Indicator plasmid with a promoter containing a SRE regulating expression of GFP (EF) | GAPS | Effectene | 365 µm | [29] |
| Comparison of different slide coatings | HEK293T | RFP EF | PS, GAPS, PLL | Effectene | 206-336 µm | [22] |
| Transfection of different cell types | a) hMSC, HEK293. b) HeLa, NIH3T3, HepG2 | EGFP and RFP EF | PLL, GAPS, and uncoated slides, FN added to transfection mix | a) JetPEI (Q-Bio Gene), TransFast (Promega) b) Lipofectamine 2000 (Invitrogen) | Not given | [20] |
| Transfection microarray of nonadherent cells | K562 | EGFP and RFP EF | BSA and BAM | Lipofectamine 2000 | 500 µm | [24] |
| Activation of a CRE-containing reporter construct by PKA in different cell types | HEK293T, N2A, SH-SY5Y, PC-12 | EGFP EF | PLL | Lipofectamine 2000, adenoviral penton protein | 700 µm | [30] |
| Characterization of binding affinities of single chain antibodies against fluorescein | HEK293T | Fluorescein BSA conjugate (EF) | GAPS | Effectene | Not given | [28] |
| High throughput cell phenotype screening with automatic phenotype recognition | MCF7 | GFP and YFP-tagged proteins (EF) | None | Effectene | 400 µm | [37] |
| Transfection of pheochromocytoma cells | PC12 | EGFP EF | Col IV | Lipofectamine 2000 and Transfectin (Bio-Rad) | Not given | [21] |
| Rapid functional annotation of uncharacterized proteins | HEK293, HeLa | Detection of exogenous proteins with V5 IF, cell characterization with antibody library against different biomarkers (IF) | PLL | Effectene | 4000 µm | [25] |
| Reverse transfection on polyvinyl alcohol coatings | HEK293 | GFP (EF) | Polyvinyl alcohol as cell-repellent surface | Effectene | 750 µm | [26] |
| Lentivirus-infected cell microarrays for gene function screens | HeLa, A549, HEK293T, DU145, BJ fibroblasts, mouse dendritic cells. | GFP and RFP EF, THY-1.1 IF | GAPS | Infection procedure | 250 µm | [23] |
| Identification of novel proapoptotic genes | HEK293T | EYFP to visualize apoptotic bodies (EF) | PLL | Effectene | 600 µm | [34] |
| Identification of proapoptotic genes | HEK293T | TUNEL | PLL | Effectene | 140 µm | [35] |
| Subcellular localization of human chromosome 21 proteins (Trisomy 21) | HEK293T | Co-localization of $His_6$-tagged exogenous proteins and organelle-specific markers (IF) | PLL | Effectene | 120 µm | [33] |
| Identification of genes associated with CRE pathway activation | HEK293T | EGFP EF | Uncoated glass slides | Effectene | 600 µm | [31] |

**(Table 1) contd…..**

| Goal | Cell Type | Reporter System | Coating | Transfection Reagent | Spot Diameter | Ref. |
|---|---|---|---|---|---|---|
| **Overexpression of proteins** | | | | | | |
| Combination effects of different transcription factors on vascular progenitor cell differentiation | EB5 | EGFP expression under the control of the FLK-1-promoter | Col IV | Lipofectamine 2000 | 1000 µm | [32] |
| Detection of antigen-specific CD8+ cytotoxic T-cells | HEK293T | GFP and rhodamine red EF | Epoxy-coated slides | Effectene | Not given | [36] |
| **Gene knockout** | | | | | | |
| Identification of effective RNAi probes | HeLa | Expression of a chimeric fusion protein of EGFP and protein of interest (EF) | GAPS | Lipofectamine 2000 | 500 µm | [38] |
| Information management of high throughput siRNA approaches | HeLa | Destabilzed EGFP EF | PLL | Lipofectin | 100-500 µm | [39] |
| Optimization of reverse transfection | HEK293T, HeLa, HaCaT | EGFP EF | PLL | Effectene | 200 µm | [27] |
| Screen for a) defects in proteosome function and b) cytokinesis | 293T, IMR90/E1A, NIH3T3, HeLa | a) GFP fused with a PEST sequence, b) α-tubulin GFP fusion construct (EF) | GAPS | Effectene (Qiagen) | 400-500 µm | [40] |
| Loss-of-function screens in drosophila cells using living-cell microarrays | $Kc_{167}$, $S2R^{+}$ | Numbers of Hoechst dye-stained nuclei/spot (proliferation), Sytox stain (apoptosis), phosphorylation of dAkt in absence and presence of dPTEN | Concanavalin A, amino non-silane | None | 200 µm | [41] |
| Microarray of lentiviruses for shRNA expression | HeLa | Phospho-S6 or lamin A/C IF | GAPS | Infection procedure | 250 µm | [23] |
| High throughput RNAi screening by time–lapse imaging of live human cells using a fully automated analysis platform | HeLa, PHSF, U-2 OS, RPE, A549 | Expression of histone (H2B) GFP to visualize chromosome segregation and structure (EF) | None | Lipofectamine 2000 | 400 µm | [42] |
| Transfection of siRNA arrays in combination with multi-channel IF and time-lapse microscopy | HeLa, PHSF, U-2 OS, RPE, A549, HUVEC | Morphology of cell nuclei stained with Hoechst dye | None | Lipofectamine 2000 | 400 µm | [46] |
| Identification of molecules involved in the secretory machinery | HeLa | Monitoring of membrane translocation of a temperature sensitive CFP-coupled viral membrane protein (tsO45G) (EF and IF) | None | None | 400 µm | [43] |

hydrophobicity [polystyrene (PS), amino silane (GAPS), poly-L-lysine (PLL)] were investigated. An increase of transfection efficiency with increasing hydrophobicity of the coating substrate was found. However, a closer examination showed that although the same pin size was used for printing, the spot size decreased with increasing substrate hydrophobicity [22]. Stronger hydrophobicity of the coating resulted in a relative increase of plasmid DNA amounts in the application area and *via* this indirectly increased transfection efficiency.

Several studies evaluated the effect of different transfection reagents. For example it has been shown that JetPEI (Qbiogene) and TransFast (Promega) produce best transfection results in human mesenchymal stem cells and HEK293 cells, whereas Lipofectamine 2000 (Invitrogen) was found to work best for HeLa, NIH3T3, and HepG2 cells [20]. An overview of coatings and transfection reagents which worked best for different groups using different cell types is given in Table **1**. Altogether, the many different procedures described indicate that for each cell type used careful standardizations of both coating and transfection reagents is required to achieve optimal results.

An elegant approach to use RTCM technology with a standardized procedure suitable for many different cell types was again introduced by Sabatini's group [23]. They demonstrated that concentrated pseudotyped lentiviruses can be used to perform lentivirus-infected cell microarrays (LICM). With this method different proteins (THY-1.1, GFP, RFP) and shRNAs (targeting mTOR or lamin A/C) driven by different promotors (phosphoglycerate promoter, ubiquitine C

promoter) could be successfully expressed in many different proliferating cell types (HeLa, HEK293T, human alveolar basoepithelial cells, human prostate carcinoma cells, human foreskin fibroblasts) and in resting bone marrow-derived murine dendritic cells. However, the authors discussed that presently the main challenge of the ambitious LICM approach to be used for genome-wide screens is the requirement for high-titer virus obtained by concentrating virus-containing supernatants.

As yet, all reports summarized used RTCM exclusively with adherent cells. A new perspective was provided when this method was successfully used with non-adherent cells. Kato and colleagues fixed human erythroleukemia cells (K562) to the slides with a biocompatible anchor for membranes (BAM) [24]. The BAM linker consists of an oleyl group as a hydrophobic cell membrane anchor and an activated polyethylenglycol group for increased water solubility and reaction with material surfaces. By using BAM as coating reagent K562 cells could be fixed with sufficient strength onto the slides allowing the uptake of liposomal-DNA complexes. In experiments where GFP- and RFP-coding plasmids were spotted 500 μm apart from each other no cross contamination occurred between the different transfection clusters.

The potential problem of cross contamination between different reversely transfected cell clusters may specifically appear when using cell types with low adhesiveness and/or high migratory activity. In this case the generation of spatially separated cell seeding areas has been suggested as a potential solution to avoid cross contamination between cell clusters. For example the use of silicon gaskets containing 50 miniature wells fitting on PLL coated slides [25] and the generation of spatially separated cell adhesion areas on the slides by particular coating [26] have been used to address this point. For the latter approach glass slides are treated with polyvinyl alcohol to provide a cell repelling surface. Then the slides are treated with sodium hypochloride, which is applied with a spotter to create defined areas for cell attachment [26]. Both approaches may provide useful alternatives to standard RTCM procedure in case of cross contamination between different samples. In this framework it has also been shown that repeated DNA application on the same spot should be avoided, because it caused leakage of the expression vectors outside of the transfection spots and spreading of transfected cells [27].

***Determination of gene effects:*** Many different innovative approaches have been described to determine the effect of cDNA molecules in reversely transfected cells. Among the first approaches were screens to determine ligand-receptor interactions on the cell surface [28] and reporter gene expression tests to detect activation of specific signal transduction pathways [29, 30]. Reporter gene expression tests used plasmids which expressed the indicator gene GFP *via* inducible promoters with either a serum response element (SRE) [29] or a cAMP response element (CRE) [30, 31]. SRE is activated by multiple signaling pathways including the mitogen-activated proten kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways whereas CRE is activated by cAMP-dependent protein kinase (PKA). It has been shown in different cell lines that both reporters can be used to detect the activation of the respective signal transduction pathways. Co-transfection of the respective activators resulted in an increased expression of GFP which was quantified with a laser scanner or a fluorescence microscope. This offers the possibility to screen libraries for the identification of proteins that are at present not known to activate the aforementioned signaling pathways as was shown by Tian and colleagues [31]. The authors identified four novel activators [ring finger protein 41 (RNF41), chromosome 8 open reading frame 32 (C8orf32), chromosome 6 open reading frame 208 (C6orf208), Meis homeobox 3 pseudogene 1 (MEIS3P1)] of the CRE pathway by screening a cDNA library containing 575 genes [31]. A reporter gene expression test was also used by Yamauchi and colleagues, who investigated combination effects of different transcription factors on vascular progenitor cell differentiation [32]. They expressed eleven relevant transcription factors alone or in combination by pairs in mouse embryonic stem cell lines. Subsequently, they scored the impact on induction of endothelial cell differentiation by quantifying the expression levels of an EGFP indicator gene under the control of the vascular endothelial growth factor receptor 2 (Flk1) promoter. In this test v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets1) and Ets1/sex determining region Y-box 7 (Sox7) combinations were identified as potent activators of endothelial cell differentiation.

For phenotypic characterization, immunofluorescent staining of different biomarkers indicating specific cellular compartments, apoptosis and activation of signal transduction pathways are most often used. A library of antibodies specific for different biomarkers to characterize cell phenotypes in RTCM has been used to investigate the effects of 46 selected virtually uncharacterized human genes [25]. With this approach a Golgi and endosome disrupting protein and a protein which triggered changes in the expression pattern of the small GTPase Rac1 and of phosphorylated histone 3 (PH3) were detected [25]. Another pathogenetically relevant study was reported by Hu and colleagues [33]. They cloned 89 genes distributed over the 21q chromosome (involved in trisomy 21) and expressed the respective proteins with an amino terminal His-tag. Organelle-specific localization was analyzed by immunocytochemical co-staining of the His-tagged proteins and organelle-specific markers for example lysosome-associated membrane protein-2 (lysosome), protein disulfide isomerase [endoplasmatic reticulum (ER)], and gamma-adaptin (Golgi apparatus) to contribute to further understanding of the functions of these proteins.

Apoptotic genes have been screened by two different studies [34, 35]. Both studies used HEK293T cells and investigated apopotic effects of 382 genes and 1.959 genes, respectively. Apoptosis induction was either measured by TUNEL analysis (detection of DNA fragmentation), co-transfection with EYFP (to demonstrate formation of apoptotic bodies) or 4',6-diamidino-2-phenylindole (DAPI) staining (to detect nuclear fragmentation). All three methods were applicable. Apoptotic body formation has been found to be most sensitive whereas nuclear fragmentation and TUNEL analysis showed lower sensitivity. The high sensitivity of apoptotic body formation was attributed to the fact that many apoptotic bodies are released from one apoptotic cell, which results in signal amplification [34]. In a recent work Rong and colleagues used RTCM to screen for antigen specific cytotoxic T-cells (CTL) [36]. An expression vector coding

for the HY-antigen fused to GFP was reversely transfected into HEK293T which stably expressed a major histocompatibility complex class I molecule and a T-cell costimulatory ligand (B7.1). Subsequently, HY-antigen specific CTL were added and induction of cell death could be detected by labeling of activated caspases with a fluorochrome (rhodamine red)-labeled inhibitor of caspases (FLICA). By using suitable cDNA libraries, this CTL array may offer a novel strategy to identify tumor or virus specific antigens.

Moreover, data evaluation and data handling are constantly improved. A highly sophisticated approach for high throughput cell phenotype screening was described by Conrad and colleagues combining human live cell arrays, screening microscopy and classification methods based on machine-learning [37]. Different GFP-tagged cDNA products were expressed in MCF7 breast cancer cells. Localization in different cellular compartments (cytoplasm, mitochondria, plasma membrane, ER, nucleoli, peroxisomes, microtubules, nucleus, and Golgi apparatus) was categorized automatically. Accuracy of recognition was 74-95% for all compartments except ER (31%), which was frequently incorrectly classified as microtubules or mitochondria. The latter is in agreement with visual similarities of these images. Specifically with computer-based self learning programs the biological variation of the same phenotype in the different cells remains the most challenging aspect.

In summary, the available publications show that the RTCM method for the analysis of gene function by overexpression of cDNAs has made significant progress in the past years. Several different cell types can be used and many different methods exist to measure the impact of the expressed gene on the cell phenotype. This progress is also reflected by the fact that an increasing number of studies addressing pathogically relevant questions have been published lately.

## RTCM TECHNOLOGY AND GENE SILENCING

***Transfection Technology***: The use of array transfection technology for high throughput RNAi applications started in 2003 [38]. The aim of one of the first studies was to provide a high throughput platform for the identification of effective siRNA and shRNA molecules against any target gene [38]. The authors used a MyoD-cDNA fused upstream of an EGFP coding sequence as a reporter and expressed this construct on an array platform in HeLa cells. The chimeric cDNA was co-transfected with different siRNA and shRNA molecules directed against MyoD. The effectiveness of MyoD knockdown was analyzed by quantifying EGFP fluorescence. This method allowed the identification of the most effective siRNA and shRNA against MyoD and the results obtained on the array transfection platform were in accordance with those obtained in conventional transfection experiments. Another group optimized the use of siRNA in RTCM with HEK293T cells [27]. It was noticed that siRNA-specific transfection reagents [Transmessenger, RNAiFect (Qiagen), and Oligofectamine (Invitrogen)] were less effective than Effectene (Qiagen) in the RTCM method. In the presence of optimized conditions, EGFP expression could be repressed by 97%. Mousses and colleagues performed a similar approach, however with the goal to optimize information management for high throughput siRNA approaches [39]. In this study, a destabilized EGFP was stably expressed in HeLa cells and targeted by rhodamine-labeled EGFP-specific siRNA. The siRNA and transfection reagents were spotted together with matrigel to confine the spread of siRNA. With this approach it was shown that both the fluorescent-labeled siRNA and the EGFP-silencing effect were not transmitted outside of the spot. This demonstrated that small molecules like siRNAs can be used in RTCM in a space restricted manner [39]. The assessment of the functional effects of RNAi was done with an automated high-resolution microscope-based image analysis system. The authors analyzed time- and dose-dependent RNAi effects to verify the results of their platform.

Finally, the expression of lamin A/C and GFP could be successfully silenced by infection cell culture arrays with lentiviral vectors. The recombinant lentiviruses efficiently infected the cells above the application spot and expressed the respective shRNA molecules [23]. As discussed above, lentiviral infection can be applied to many diffeent cell types, but is hampered by the requirement of high-titer virus preparations.

***Determination of gene effects:*** Several specific approaches have been described to determine the effects of the siRNA-mediated gene silencing on the cell phenotype. Silva and colleagues established a method to screen for defects in proteasome function and in cytokinesis [40]. For the first approach, GFP was fused to a sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST sequence) which targets the protein for degradation by the proteasome or by calpains. Using 30 different shRNAs directed against proteasome components they could determine that the GFP-PEST protein was most stable and accumulated in the cell when the 19S subunit of the proteasome was targeted. To screen for alterations in the cytokinesis they expressed an α-tubulin-GFP fusion protein. This protein labeled the microtubules of the respective cells. Inhibition of the mitotic motor protein Eg5 by shRNA caused aberrant cytokinesis which was detectable by the formation of characteristic "rosette"-like structures consisting of labeled microtubules with DNA at the periphery [40].

Thitherto, the RNAi RTCM approaches were proof-of-concept work and it had to be demonstrated that the parallel knockdown of multiple endogenous genes may be successfully carried out in the array transfection method. The largest published RNAi cell microarray to date was reported by Wheeler *et al.* in 2004 in cultured *Drosphila melanogaster* cells [41]. Using high-resolution automatic image acquisition and analysis they investigated the effects of 384 different genes on cell proliferation, nuclear size and dAkt phosphorylation. Proliferation was determined by counting the numbers of Hoechst dye-stained nuclei per transfection spot, apoptosis by Sytox staining (a fluorescent molecule that stains only nuclei of dead cells), and phosphorylation of dAkt in absence or presence of dPTEN (the Drosophila homolog of the human PTEN tumor suppressor that represses the PI3K/Akt pathway) by immunofluorescence [13, 41].

Genome-wide screens of gene functions with siRNA libraries require elaborated procedures of data recording and processing. Recently, two studies reported the use of fully automated procedures for phenotyping digital images in a high throughput work flow by time-lapse microscopy on cells transfected with siRNA [42, 43]. In the first study 49

genes with known or predicted functions in chromosome segregation or nuclear structure organization were targeted. The acquired images were analyzed using a custom-made software after fully automated high throughput time-lapse microscopy. This software was able to distinguish between the categories interphase, mitosis, cell shape, and apoptosis. The dynamic range of the phenotype penetrance could be successfully determined for all of these genes with the fully automated time-lapse microscopy procedure [42]. In the second study the role of different proteins involved in the secretory machinery was investigated. To this goal 37 proteins which have been shown to localize to membranes of the secretion pathways or cytoskeleton elements were targeted by different siRNAs. Subsequently, it was evaluated whether the knockdown of the respective gene affected the translocation of a temperature-sensitive CFP-coupled viral membrane protein (tsO45G) from the ER through the Golgi to the cell surface. Seven of the proteins were identified as important for the correct delivery of tsO45G to the cell surface with this approach [43].

## PROTOCOLS FOR PROTEIN OVEREXPRESSION AND GENE SILENCING IN RTCM

RTCM was predominantly designed for genome wide analysis of gene functions. The requirement of huge cloning capacity and funding may have hampered the broad application of this fascinating technology. However, RTCM also has interesting perspectives to study functional aspects of smaller genomes such as of infectious agents and of preselected groups of genes such as tumor/tumor stage-associated gene clusters obtained by microarray analysis.

In our laboratory RTCM is used as a systems biology approach in order to investigate combination effects of genes encoded by human herpesvirus-8 (HHV-8), also termed Kaposi`s sarcoma associated herpesvirus. HHV-8 is the causative agent of Kaposi´s sarcoma, an endothelial cell-derived tumor, and of two lymphoproliferative disorders, multicentric Castlemann´s disease and primary effusion lymphoma [44, 45]. HHV-8 is a member of the γ-herpesvirus family and encodes for at least 86 proteins [44, 45]. At present it is essentially unclear which genes are regulating the development of HHV-8-associated tumors.

We are specifically interested in combination effects of HHV-8 genes on different signal transduction pathways. Thus RTCM was established using indicator plasmids which express GFP under the control of different promoters which are sensitive to different signal transduction pathways such as nuclear factor-kappa B (NF-κB), p53, and activating protein–1 (AP-1) (Fig. **2A**). The indicator plasmids in combination with expression plasmids of different HHV-8 genes, transfection solution (Lipofectamine 2000) and gelatine were combined in 384-well plates (Fig. **2A**). Subsequently, these transfection mixtures were printed in duplicates onto GAPS II coated slides using solid pins (diameter 600 μM, spot center-to-center distance 1120 μm) with a contact printer (Fig. **2B**). HEK293T cells were seeded onto the printed and dried chip in a density of 1 x $10^5$ cells/$cm^2$ and were maintained for 48 hours until confluency was reached (Fig. **2C**, left). Transfection efficiency was almost 80% as determined with a plasmid constitutively expressing GFP (Fig. **2C**, right). Signal evaluation of the transfection array was carried out with a laser scanner (FLA-5000, Fuji, Fig. **2D**, left). In the presence of different HHV-8 cDNA molecules different expression levels of the GFP indicator gene were observed (Fig. **2D**, left). Quantitative evaluation of GFP signal intensities was carried out using a commerically available software (Advanced Image Data Analysis (AIDA), Raytest).

The results obtained in a single experiment were sorted according to decreasing signal intensity (Fig. **2D**, right) or inter-array comparison of different RTCM experiments was depicted in a heat map (Fig. **2D**, right).

Thirty control transfections were carried out on each slide. Ten transfections contained the plain indicator plasmid only to determine the background reactivity (Fig. **2D**, right, blue line), 10 transfections the plasmid expressing GFP constitutively under the control of the cytomegalovirus (CMV) promoter to generate strong signals as positional fixed points and finally 10 transfections the indicator plasmid in combination with a known activator of the respective signal transduction pathway to determine the sensitivity of the indicator system to the respective signal transduction pathway. The specific methodology is described in Fig. **3**.

The critical steps according to our experience are: (1) Opti-MEM/sucrose and the gelatine solutions have to be prepared freshly. (2) The same batch of Lipofectamine 2000 has to be used for the whole experiment if series of several different chips are transfected. (3) Samples in the spotter plate have to be mixed in an identical manner. (4) Evaporation must be reduced to a minimum by storing the master plate at 12 °C during the printing process. (5) Printed and dried slides should be stored protected from moisture and dust if intended for later use.

Another approach has been designed for the testing of genome-wide siRNA libraries. In this case spotting is carried out directly into LabTek tissue culture chambers (Fig. **4A**,**B**). Each LabTek chamber contains 384 different samples (Fig. **4B**,**C**). Four LabTek chambers can be mounted onto a microscope holder for the evaluation of signals with an automatic microscope (ScanR, Olympus, Biosystems), (Fig. **5A-C**). Several approaches are available for the evaluation of the effects of siRNA-mediated gene silencing on the cellular phenotype. First, phenotypic variations can be demonstrated by immunostaining (staining of the cytoskeleton, Golgi complex and ER) on paraformaldehyde (PFA)-fixed cells. Second, time-lapse experiments studying cell cycle progression or mitosis can be used. Specifically the latter approach requires sophisticated technical instrumentation and automatic analysis demands stringent controls of siRNA activity and phenotypes induced. In general, 16 control siRNAs are distributed on each LabTek. This includes negative controls (scramble siRNA, not targeting any gene), which do not affect cell growth, mitosis or protein secretion. Moreover, three positive controls are used which induce clearly visible phenotypes. The array of the siRNA transfection is recorded as successful only when these phenotypes are clearly detectable. The following positive control siRNAs has been found to be useful: (1) A siRNA targeting INCENP (inner centromere protein), which induces butterfly-like shaped nuclei in the transfected target cells and (2) an siRNA targeting KIF11 (kinesin family member 11). The respective cells show a pro-metaphase arrest. (3) A siRNA targeting COPB (coat

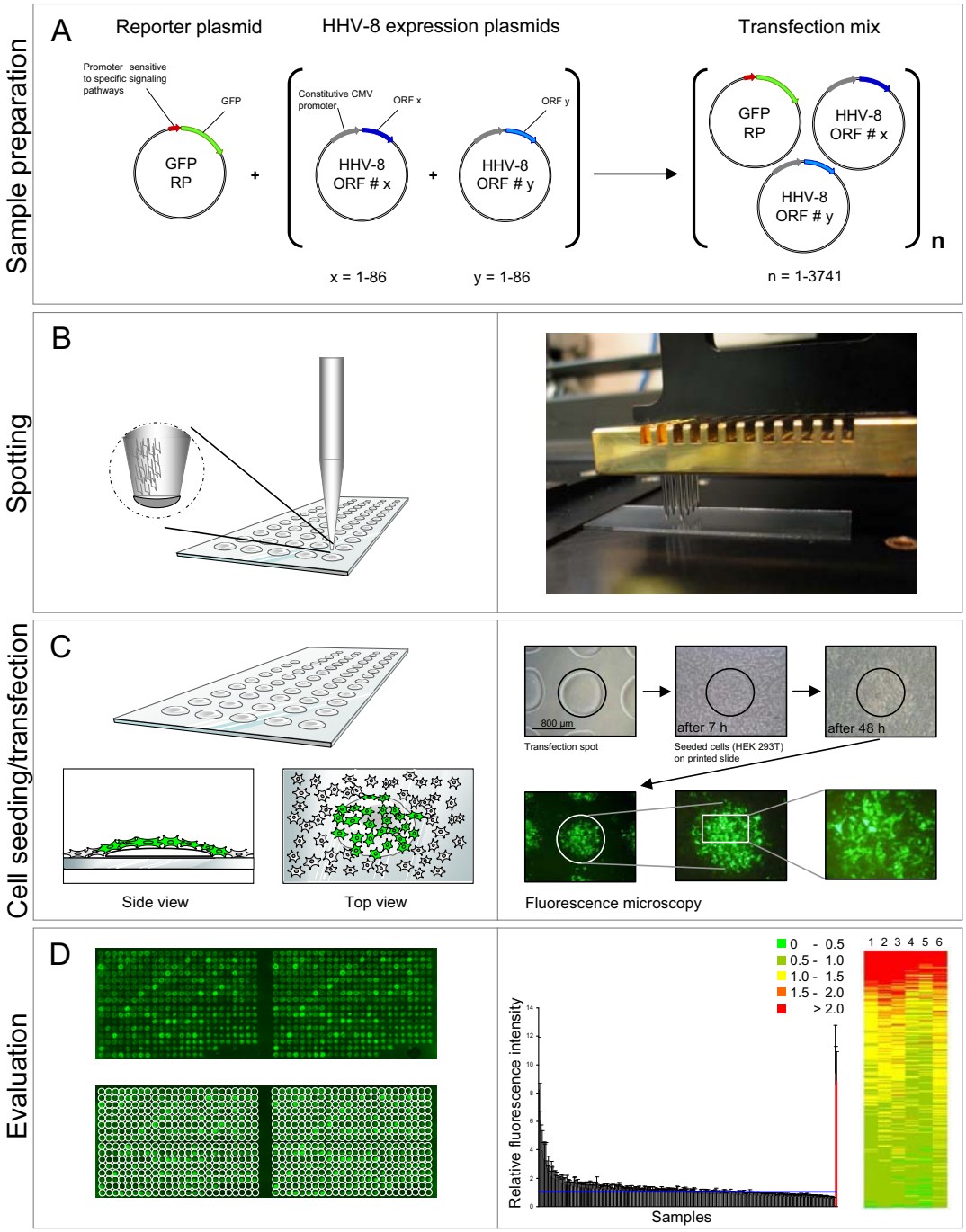


**Fig. (2). High throughput analysis of signaling activity of overexpressed proteins.** (**A**) Transfection mixtures containing a reporter plasmid expressing GFP under the control of signal transduction pathway sensitive promoters (e.g. NF-κB, p53, AP-1) in combination with one or two different plasmids expressing the HHV-8-encoded genes are manually mixed with gelatine and transfection reagent in 384-well plates. The combination of all 86 HHV-8 genes results in 3741 different transfection mixes. (**B**) Subsequently, the plate is cooled to 12 °C and spotting is carried out with a contact printer (VersArray ChipWriter Pro) using solid pins (PTS 600) with a tip diameter of 600 µm and a center-to-center distance of 1120 µm. (**C**) HEK293T cells are seeded onto the printed chips [left: schematic drawing; right: experimental data (printed spot is encircled)]. Initial cell density was 1 x $10^5$ cells/$cm^2$ (right, 7 h, phase contrast picture) to reach full-confluency after 48 h (right, 48 h, phase contrast picture). Fluorescence microscopy shows that almost 80% of the cells express GFP on a transfection spot with a control plasmid constitutively expressing GFP. (**D**) Signal detection is carried out with a laser scanner (FLA-5000) with a resolution of 25 µm (left, upper). Each transfection pattern is reproduced twice on every chip. Highly reproducible signal patterns are obtained (left). Individual detection areas are recognized by a grid mask (left, lower). Obtained signals are analyzed with a quantification software (AIDA) and can be sorted according to increasing or decreasing signal intensities or may be presented in a heat map (right). The blue line indicates the relative mean fluorescence intensity of 10 control transfections with the indicator plasmid only. Red bars indicate signal intensities measured on transfected spots with constitutive GFP expressing plasmids. Heat map: color-coded expression levels measured in 371 transfections with different activators of a signal transduction pathway in 6 independent RTCM experiments.

A

**Sample Preparation**

Mix 3 µl of OptiMEM (Invitrogen) containing 0.4 M sucrose, 1.5 µg DNA (in 5 µl $H_2O$) and 3.5 µl of Lipofectamine 2000 (Invitrogen) in a 384-well plate.

↓

Incubate 20 min (RT).

↓

Add 7.25 µl of 0.2% (w/v) gelatine (Sigma) solution and mix carefully.

B

**Spotting**

Cool plate to 12 °C during printing.

↓

Print onto GAPS II coated slides (Corning) with solid pins (tip diameter 600 µm, spot center to center distance 1120 µm).

↓

Dry slides at RT in a box with drying pearls (Neolab) for at least 12 h.

↓

Dried slides can be stored for months.

C

**Cell seeding / Transfection**

HEK293T cells are grown at 37 °C and 8.5% $CO_2$ in DMEM with 100 U/ml penicillin and 100 µg/ml streptomycin (all PAA).

↓

Split cells ~48 h before seeding 1:8 to obtain a 70-80% confluent layer of actively growing cells at the day of the reverse transfection experiment.

↓

Seed $1*10^5$ cells per $cm^2$ on the printed slide in a culture dish.

↓

Cultivate cells to confluence (~40-48 h).

D

**Evaluation**

Fix cells with buffered 4% Paraformaldehyde (pH 7.2).

↓

Determine GFP signals with a laser-scanner (e.g. FLA-5000, Fuji).

↓

Quantify signals with software (e.g. AIDA, raytest).

**Critical steps**

- Prepare OptiMEM, containing 0.4 M sucrose and gelatin solutions freshly.
- Use the same batch of Lipofectamine 2000 for the whole experiment.
- Mix every sample in the same way.
- Place the 384-well plate at 12°C while printing to avoid evaporation.
- Store the printed slides after printing in a box with drying pearls.

**Equipment**

- Contact Printer: VersArray ChipWriter Pro (Bio-Rad)
- Solid pins: PTS600 (Point Technologies)
- Laser Scanner: FLA-5000 (Fuji)

**Fig. (3). RTCM protocol for protein overexpression.** The method is divided into four parts: (**A**) sample preparation, (**B**) spotting, (**C**) cell seeding/transfection, (**D**) evaluation. Critical steps for the method according to our own experience as well as the minimal equipment required are given.

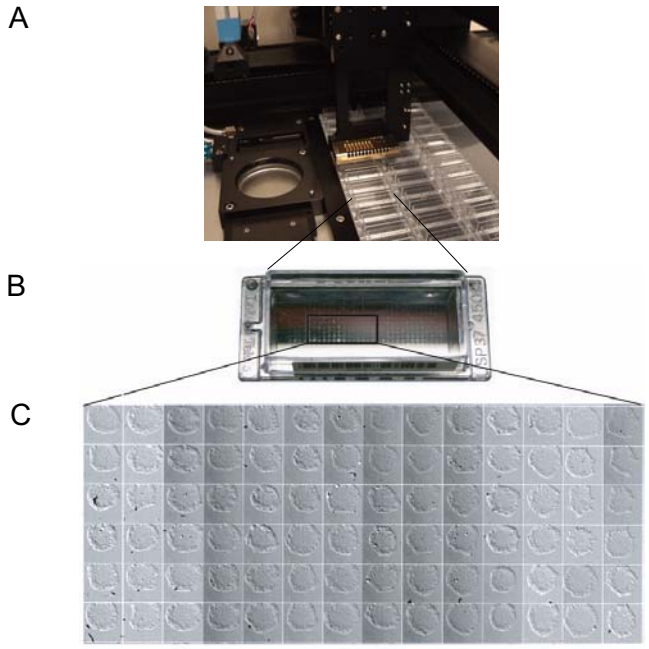


**Fig. (4). High throughput screening of genome-wide siRNA libraries.** (**A**) The contact printer applies different siRNA samples complexed with transfection solution into LabTek chambers (384 different siRNAs per chamber, 48 chambers per spotting). (**B**) Each LabTek chamber contains 384 transfection spots. (**C**) Microscopic evaluation of the dried spots shows highly regular transfection points. Adapted with modification from [46].

protein, subunit beta 1) which leads to the inhibition of protein secretion from the cells and induces apoptosis, which can be seen by nuclear fragmentation. Cells are stained with Hoechst 33342 to visualize cell nuclei. In order to detect successfully transfected cells rhodamine coupled siRNA can be used. The detailed protocol for the siRNA procedure in RTCM has been described previously [46].

## CONCLUSION

RTCM allows the parallel analysis of gene effects on the phenotype of mammalian cells in a high throughput manner. The method has been used both to investigate the effects of gene overexpression and of gene silencing. It has been successfully applied in studies with several different cell types. Innovative methods have been introduced to allow quantitative determination (1) of the effects of many different genes on cell phenotypes and (2) of the specific features of the respective gene products, such as intracellular localization and secretion. Surprisingly, despite all the advances made in this technology since its first description in 2001, the number of publications using RTCM is still quite limited. In addition, many of these papers addressed proof-of-principle topics and were conducted by large research institutes which are approaching genome-wide screens of gene function. Apparently, genome-wide approaches require sophisticated instrumentation and adequate financial resources, which may have hampered the broad application of this technology. However, recent reports as well as our own experience suggest that RTCM may also provide useful perspectives for smaller laboratories. In this framework the analysis of combination

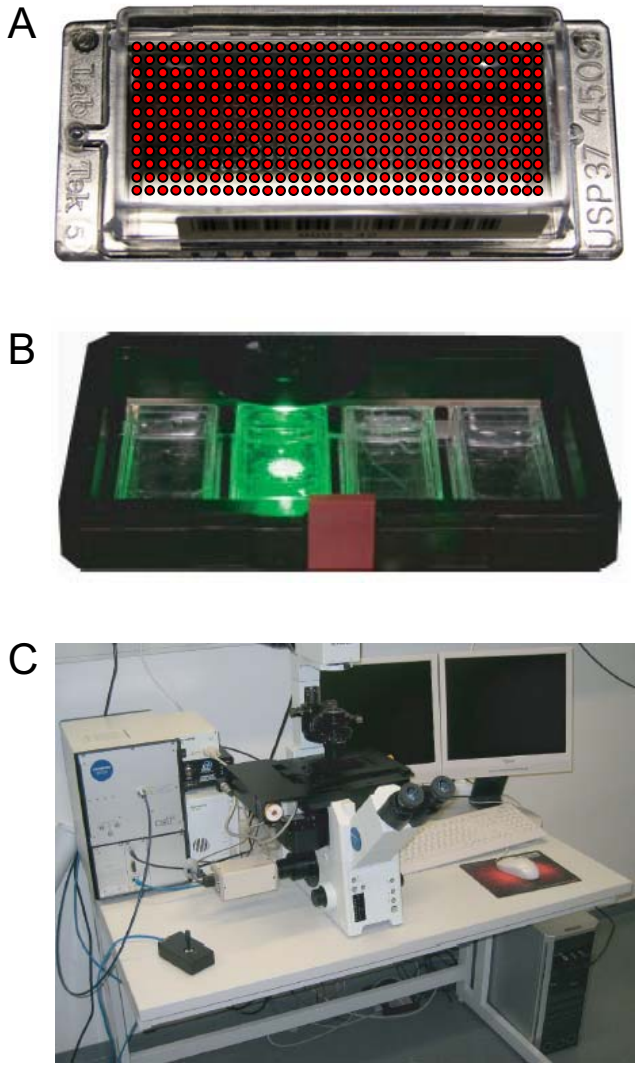


**Fig. (5). Automatic microscopic evaluation of siRNA transfection arrays.** (**A**) LabTek chambers containing 384 different samples are positioned in a microscope holder, (**B**) fitting 4 LabTek chambers (1536 samples). (**C**) Signals are analyzed in an automatic microscope (ScanR, Olympus, Biosystems). Adapted with modification from [46].

effects of limited numbers of genes extracted from biologically meaningful samples, such as infectious agents (viruses and bacteria) or from pathogenetically relevant gene clusters found to be co-expressed by transcriptome analysis may be worthwhile targets to be considered. In particular, RTCM may be used here without the need of extensive cloning and sophisticated instrumentation for data processing.

It is clear that genome-wide applications will be restricted to certain well-equipped and well-funded institutions. For this approach time-lapse microscopy in combination with automated data acquisition and procession provides the most challenging goal allowing parallel analysis of the dynamics of gene activity in mammalian cells in a high throughput fashion. This technology most likely will significantly improve drug screening procedures and the elucidation of gene function in mammalian cells in future.

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## ABBREVIATIONS

A549 = Human alveolar basal epithelia cells

AP-1 = Activating protein-1

BAM = Biocompatible anchor for membranes (oleyl-O-poly(ethylene glycol)-succinyl-N-hydroxy-succinimidyl ester)

BJ fibroblasts = Human foreskin fibroblasts

BSA = Bovine serum albumin

CFP = Cyan fluorescent protein

Col = Collagen

CRE = cAMP response element

CTL = Cytotoxic T-cells

DIGE = Difference gel electrophoresis

DNA = Desoxyribonucleic acid

(c)DNA = Complementary DNA

dPTEN = Drosophila homolog of the human PTEN tumor suppressor that represses the PIsK/Akt pathway

DU145 = Human prostate carcinoma cells

EB5 = Mouse embryonic stem cell line

EF = Epifluorescence

ER = Endoplasmatic reticulum

FLK-1 = Vascular endothelial growth factor receptor-2

FN = Fibronectin

GAPS = Amino silane

(E)GFP = (Enhanced) green fluorescent protein

HaCaT = Human skin epithelial cell line

HEK293 = Human embryonic kidney cells

HEK293T = Human embryonic kidney cells expressing SV40 large T antigen

HeLa = Cervix carcinoma cell line

HepG2 = Human hepatocellular carcinoma cell line

hMSC = Human mesenchymal stem cells

HUVEC = Human umbilical vein endothelial cells

IF = Immuofluorescence

IMR90/E1A = Human fibroblasts expressing adenoviral E1A antigen

JNK = c-Jun N-terminal kinase

K562 = Human erythroleukemic cell line

$Kc_{167}$ = Drosophila embryonic cells

LICM = Lentivirus-infected cell microarrays

MAPK = Mitogen-activated protein kinase

MCF7 = Breast cancer cells

N2A = Mouse neuroblastoma cell line

NIH3T3 = Mouse embryonic fibroblast cell line

NF-κB = Nuclear factor-kappa B

PC12 = Human pheochromocytoma cells

PEST = Proline-glutamic acid-serine-threonine-protein sequence

PHSF = Primary human skin fibroblasts

PKA = cAMP-dependent protein kinase

PLL = Poly-L-lysine

PS = Polystyrene

RFP = Red fluorescent protein

RISC = Ribonucleic acid-induced silencing complex

RNA = Ribonucleic acid

mRNA = Messenger RNA

(mi)RNA = Micro ribonucleic acid

(sh)RNA = Short hairpin ribonucleic acid

(si)RNA = Short interfering ribonucleic acid

RNAi = Ribonucleic acid interference

RPE = Human retinal pigment epithelial cells

RTCM = Reversely transfected cell microarrays

$S2R^{+}$ = Schneider S2 embryonic drosophila cells

SH-SY5Y = Human neuroblastoma cell line

SRE = Serum response element

TAP = Tandem affinity purification

THY-1.1 = Thymocyte differentiation antigen 1 (CD90a)

TUNEL = Terminal deoxynucleotide transferase dUTP nick end label assay

U-2 OS = Human osteosarcoma cell line

(E)YFP = (Enhanced) yellow fluorescent protein

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