3. Methods

3.1. Plasmid construction

The construction strategy was based on Invitrogen Gateway™ cloning technology, which utilizes the site-specific recombination properties of bacteriophage lambda. Gateway™ cloning technology constitutes two essential recombinant reactions (i.e. BP and LR reaction) that are directed with so-called *att* sites. In this study, *att*B-site containing PCR primers were designed and used to amplify the sequences of Chr21 genes. BP reactions were performed with *att*B-PCR products and a donor vector to generate "entry" clones. Through LR reactions, gene sequences in the entry clones were transferred again into destination vectors to generate expression (pEXP) clones.

3.1.1. ORFs amplification with *att*B-PCR primers

Known and predicted Open Reading Frame (ORF) sequences were retrieved from the Chromosome 21 gene catalogue (http://chr21.molgen.mpg.de/chr21_catalog9.html). The cDNA clones used for PCR amplification were obtained from Human MTC Panels I+II and QUICK-Clone cDNA (Clontech, Heidelberg, Germany) or from public IMAGE or MGC cDNA clones (RZPD, Berlin, Germany). The primer pairs were designed using PRIDE (http://pride.molgen.mpg.de/pride.html). Two sets of primers—template-specific primers and *att*B adapter primers—were designed to generate the *att*B-PCR products. The template-specific primers were used for the first-step PCR to amplify the full-length of ORFs. For the construction of N-terminal tagged expression vectors, the start primers were designed with the ATG codon and the stop primers were designed with or after the termination codon. For the construction of C-terminal tagged expression vectors, the start primers were also designed with the ATG codon, whereas the stop primers were designed to target the gene sequence before the termination codon. In the second-step PCR, the *att*B adapter primers (see Table A.2 in Appendix for the sequence) were used to install the complete *att*B sequences into the ends of PCR products. The primers used for the amplification of Chr21 genes were listed in Appendix (Table A.1) and PCR amplification was carried out following Gateway™ technology handbook. The high fidelity Platinum® Pfx DNA polymerase (Invitrogen) was used for all ORF

amplification. After agarose gel electrophoresis, the *att*B-PCR products of correct sizes were purified as described in Gateway™ technology handbook.

3.1.2. Construction of pENTRY clones via BP reaction

The *att*P-containing donor vector pDONR™201 (Invitrogen) was used to generate the "entry" clones through BP recombination with *att*B-PCR products, a reaction catalyzed by BP Clonase™ enzyme mix (Invitrogen). Each reaction was set at 10 µl of the total volume, and the right amount of reaction components were calculated following Gateway™ technology instruction manual. DH5α™ competent cells (Invitrogen) were used for the transformation of BP reaction, and the positive clones with the correct identities of the inserts were verified by generation of 5′ and 3′ ORF sequence tags (OSTs). The resulted "entry" clones contained the *att*L sequences and were ready for creating expression clones via LR recombination reaction.

3.1.3. Construction of expression plamsids via LR reaction

The "entry" clones coding for 89 Chr21 proteins and localization control proteins were incubated with pDEST™26 vectors (Invitrogen) in the presence of LR Clonase™ enzyme mix (Invitrogen) to create mammalian expression clones. pDEST™26 vector carries a 6×-Histidine (His_6) tag at the N-terminus to the protein-coding sequences, which allows the detection of all expressed recombinant proteins by using an anti-His antibody. To generate C-terminal tagged expression clones, pDEST474 (a kind gift from Dominic Esposito, NCI-Frederick Vector Engineering Group, USA) was used to include a Myc-tag to the C-terminus of recombinant proteins. DH5α™ and DH10B competent cells (Gibco life technologies, Invitrogen) were used for the transformation of LR reactions. The positive clones were screened firstly by PCR, and identities of the inserts were verified by sequencing.

Table 3.1 Primer pairs used for gene identity confirmation by PCR and sequencing

Expression clone	Primers
pEXP26	forward: 5' - CGG ACC ATG GCG TAC TAC C - 3'
	reverse: 5' - TAA TAC GAC TCA CTA TAG GG - 3' (T7_forward)
pEXP474	forward: 5' - GCCTGCAGGTACCGGATCA - 3'
	reverse: 5' - G CTT TTG TTC GCT AGC CAT TC - 3'

Table 3.2 Standard PCR temperature profile and PCR mix

Step	Temperature	Time	Cycles	PCR mix (50 µl reaction)
Initial denaturation	94 °C	3 min	1	5 µl template colony in H20
Denaturation Annealing Extension	94 °C 45 °C 72 °C	30 sec 30 sec 2 min	30	5 µl 10 x PCR Buffer (RocheApplBiosys) 1 µ l dNTP Mix (10 mM each) 0.8 µ l Primer Mix (25 µM each)
Final extension	72 °C	5 min	1	0.3 µl Taq Polymerase (10 U/µl, house-made)
Storage	4 °C	infinite	1	37.9 µ l H2O

3.2. Transfected Cell arrays

Transfected cell arrays were prepared following three steps: “Probe” (DNA or siRNA samples) preparation; array printing; and reverse transfection of spotted arrays into cells.

3.2.1. Probe preparation

Two methods called 'gelatin' method and 'lipid' method were used to prepare DNA or RNA sample for microarray printing and reverse transfection. In the 'gelatin' method, DNA or RNA sample in gelatin solution were firstly arrayed on the glass slides and the transfection reagent was added to all the samples just before the transfection. In 'lipid' method, the lipid-based transfection reagent was pre-mixed with the DNA or RNA before spotting.

Materials

- Endotoxic-free maxi-preparation kit; Miniprep kit (Qiagen)
- 0.2 % (for 'gelatin' method) and 0.05% (for 'lipid' method) Gelatin solution (see below) (Sigma)
- Effectene, RNAiFect, and HiperFect Transfection Reagent Kit (Qiagen)
- EC buffer (from EffecteneTransfection Kit) containing 0.2-0.4M sucrose (Invitrogen)
- siRNA suspension buffer (Qiagen)

Preparation of 0.2% and 0.05% (w/v) Gelatin Solution

1) Dissolve gelatin powder in sterile MilliQ water by gently swirling mixture for 15 minutes in a 60ºC water bath.
2) Cool the 0.2% gelatin solution at room temperature, and, while still warm (~37-40ºC), filter it through a 0.45 µm cellular acetate membrane (CA). Store the aliquots of filtered gelatin solution at 4ºC before use.
3) For 0.05% gelatin solution: dilute 0.2% gelatin solution with sterile water to final concentration of 0.05%.

DNA plasmid preparation

Qiagen Miniprep Kit was used to prepare most of the expression plasmids, following the manufacture instruction except increasing the bacterial culture from 5 ml to 8 ml for each preparation. The resulted DNA concentration of Chr21ORFs plasmids was generally between 0.4 ~ 0.8 µg/µl. For the particular genes whose yields were below 0.4 µg/µl, Qiagen Endotoxic-free maxi-preparation kit was used following the product instruction.

siRNA stock solution preparation

The ordered siRNAs from Qiagen are lyophilized in the tube with the amount of 5 nanomol. To prepare the stock solution at 200 µM, the content of each tube was dissolved in 25 µl of the siRNA Suspension Buffer (Qiagen) by heating the tube to 90°C for 1 minute and incubating the tube at 37°C for 60 minute. The heating-up procedure is to disrupt higher aggregates, which may have formed during the lyophilization process. The entire preparation process was carried out in a RNAse-free environment and the resulted 200 µM siRNA stocks were stored at -20°C before use.

3.2.1.1. Gelatin-DNA method

To prepare each Gelatin-DNA sample for printing, in a 1.5ml sterile tube or a 96-well plate, each purified plasmid DNA was diluted, by 10-times fold, with 0.2% gelatin solution to a final DNA concentration of 0.04 ~ 0.07 µg/µl. Other dilution times more than 10-times fold were ever used, however, the final concentration of gelatin has to be remained lower than 0.2% and greater than 0.17%. After dilution, the DNA-gelatin samples were mixed and stored at 4°C before printing.

Gelatin-siRNA method was ever fulfilled for reverse transfection of siRNA on cell arrays. Before sample printing, the 200 μM siRNA stock was firstly diluted with siRNA Suspension Buffer to 50 μM and then diluted again at 10- or 20-times fold with 0.2% gelatin solution. The printed siRNA spots were treated with siRNA transfection reagent and reverse transfected into HeLa cells. This gelatin-siRNA method, however, was abandoned because the siRNA spots were observed to diffuse and mix with each other shortly after the transfection.

3.2.1.2. Lipid-DNA/lipid-siRNA method

In “lipid” method, the lipid-based transfection reagent ('lipid') was pre-mixed with the DNA or siRNA before printing. It was used in this study for the transfection of siRNAs and a few Chr21ORF plasmids whose transfection efficiency appeared to be low by “gelatin” method.

Lipid-DNA preparation protocol

1) In a 1.5 ml tube, add 1~ 2 μg DNA to 15 μl of DNA-condensation buffer (Buffer EC from Effectene Kit) in which the filter-sterilized sucrose (Invitrogen) has been dissolved to a concentration of 0.2- 0.4M.
2) Add 1.5 μl of Enhancer solution (from Effectene Kit), mix the tube contents by pipetting up and down five times, and incubate the mixture at room temperature for 5 minutes.
3) Next add 5 μl Effectene transfection reagent (from Effectene Kit), mix the solution by gentle vortexing.
4) Incubate at room temperature for 10 minutes.
5) Add a 1X (24μl) volume of 0.05% gelatin, remix the solution and store it at 4°C before printing.

Lipid-siRNA preparation protocol

1) Dilute the 200 μM siRNA stock with siRNA Suspension Buffer to 50 μM
2) Add 1 μl of 50 μM siRNA to 3 μl EC-Buffer that contains 0.2- 0.4 M sucrose (the same buffer used in 'lipid-DNA' preparation).

3) Add 4 µl of siRNA transfection reagents (RNAiFect or HiperFect, Qiagen), mix the contents by pipetting up and down five times, and incubate the mixture at room temperature for 10 minutes.
4) Add 2 µl of 0.05% gelatin, remix the solution and store it at 4°C before printing.

3.2.2. Array printing

Depending on the number of samples need to print in each test, manual spotting or robotic spotting were applied. For robotic spotting, both the arrayers utilizing solid contact pins and nano-dispensing technologies were used. By either of the printing methods, the DNA or siRNA probes were printed onto the glass slides coated with Gamma-Amino Propyl Silane (GAPS slides, Corning) or the slides treated with poly-L-lysine (see Appendix for preparation details). After printing, the glass slides were air-dried and stored in a Vacuum Desiccator with Stopcock 250 mm, NALGENE (VWR) at room temperature before reverse transfection. For a long-term use of the slides printed with 'lipid' samples, storage at 4 °C was necessary to maintain the effects of transfection reagents.

Materials

- Gamma-Amino Propyl Silane (GAPS) slides (Corning)
- Standard microscopy slides 76 × 26 × 1 mm (Menzel-Glaeser, Germany)
- Poly-L-lysine (Sigma)
- Virtek Chipwriter robotic arrayer (Virtek, Toronto, Canada)
- Stealth Micro Spotting Pins (SMP4) (Telechem International, Inc.)
- Piezo Dispenser sciFLEXARRAYER S5 (Scienion)
- Plastic pipetting tip for manual spotting (SAFESEAL tips PREMIUM XL, 10 µl. Biozmy)
- 384-well plates, “V” shape (Genetix Limited, UK)

3.2.2.1. Solid pin-contact printing

The spotting procedure was carried out using Virtek Chipwriter robotic arrayer and Telechem’s Stealth pins (type SMP4) in a hood (Fig. 3.1 A). The 'gelatin-' or 'lipid-' prepared DNA samples were transferred to a 384-well plate (“V” shape) before printing. The robot was programmed to transfer the samples to the slides by touching

the pins to the slide surface for 25-50 ms (Fig. 3.1 B). Using SMP4 pins, the printed spots have diameters of 120 µm and a distance of 400 µm between spots was usually used. The pins were washed thoroughly between each dip into a new well of samples and the entire printing hood was set at 55% humidity. The thorough wash step and 55% relative humidity environment were important to prevent clogging of samples in the arraying pins. Each Chr21 gene was spotted at least in triplicate, while GFP and/or HcRed genes were spotted along with each row and column to facilitate the observation of printing grids. Figure 3.1 C showed one spotting grid of a dried 9 × 8 spots that was taken with a light microscope.

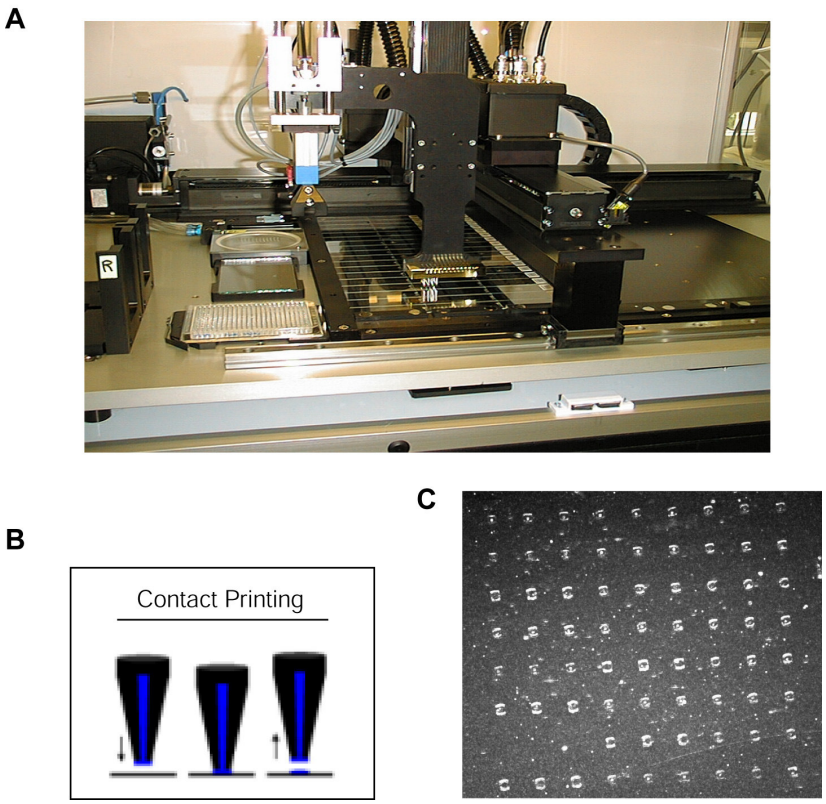

Fig. 3.1 Solid pin-contact printing of cell arrays

(A) The spotting hood used in this study, which contains Virtek Chipwriter robotic arrayer and Telechem's Stealth pins (type SMP4). (B) Schematic view of contact printing. (C) A spotting grid of 9 × 8 spots under light microscopy. See text for more details.

3.2.2.2. Flexarray dispensing printing

Compared to solid pin-contact printing, microdispensing technique was more flexible to print sample spots with preferred diameters and volumes. The print head is formed as a nozzle array; once filled with samples, the print head releases a single droplet

from each nozzle to the surface of slides without virtual contact to the slides (Fig.3.2 A). By adjusting the number of "drops" that compose the dispensing droplet, samples can be printed with different volumes and the diameters of spots varied (Fig. 3.2 B). When using Piezo Dispenser sciFLEXARRAYER S5 (Scienion) in this study, 2.5-drops droplet contains 1 nanolitre sample, while 20 drops droplet resultes in a printing spot with 400 µm micrometer. The center-to-center distance between spots was normally set at 2 mm for siRNA arrays.

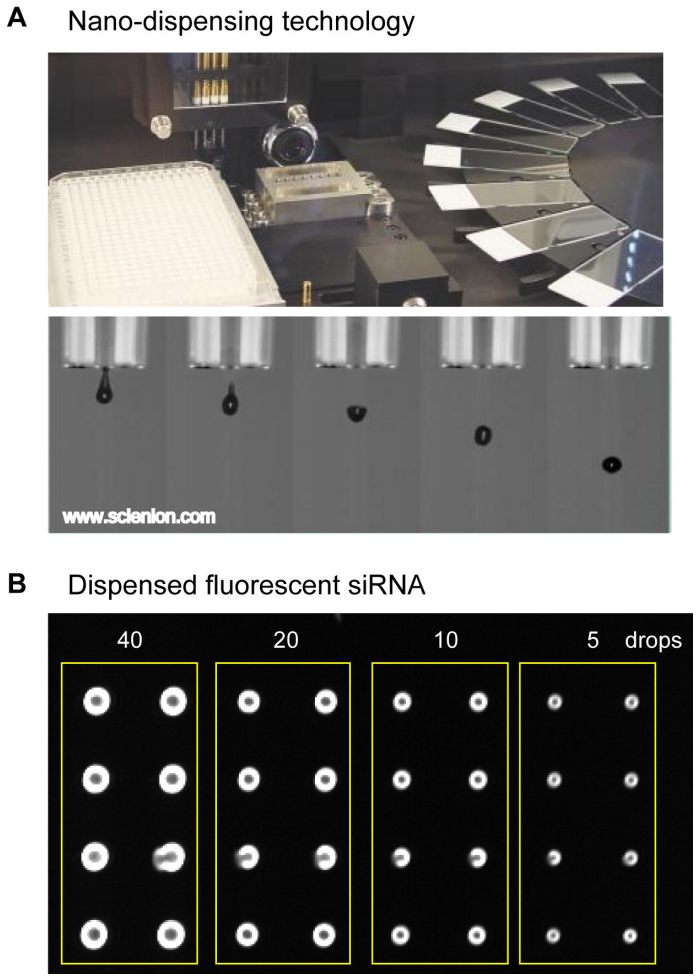

Fig. 3.2 Dispensing printing of cell arrays using Flexarray

(A) The spotting hood used in this study, and a dispensing process from a single print head (www.scienion.com). (B) Four spotting grids of 2 × 4 spots of Rhodamine-labeled siRNA. With different number of spotting "drops", arrays were produced with different volumes of samples and diameters.

3.2.2.3. Manual printing

In some testing experiments, manual printing was a useful alternative to robotic arrayers to print small number of samples, efficiently and quickly. To guide the position of printing, a spotting grid with desired size and distance was printed on a piece of paper or on a transparent slide. The glass slides ready to spot were put on top of the spotting grids, so that the spots could be printed following the grids. In this study, I normally designed spotting grids with 1.5 × 1.5 mm squares for each square. The ready-to-print DNA or siRNA samples were spotted at the center of each square without surpassing the lines, hence were separated from the adjacent spots. An example of the printing grid with 19 × 10 spots was shown in Fig.3.3.

The spotting procedure was carried out in a sterile cell culture hood. Guided by the spotting grid, the samples were printed by quickly touching the long pipeting tips (SAFESEAL tips PREMIUM XL, Biozmy) that were filled with 1 µl sample on the surface of cell array slide (Fig. 3.3). By using this type of tip, each spot contained 10 ~ 15 nl of the samples, and a batch of slides were spotted using a same tip for each sample.

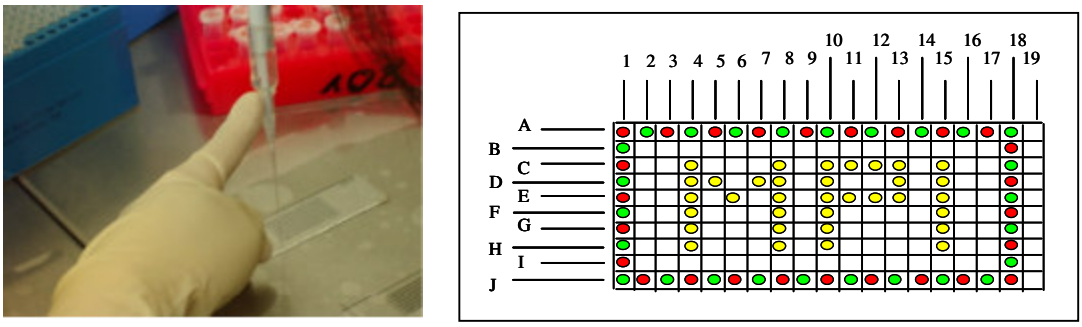

Fig. 3.3 Manual printing of cell arrays

Sample solutions were spotted by quickly touching the long pipeting tips (SAFESEAL tips PREMIUM XL, Biozmy) on the surface of cell array slide (left), which was laid on top of a printing grid (right).

3.2.3. Reverse transfection

In this step, cells were added on top of the slides printed with “gelatin-” or “lipid-” treated DNA and/or siRNA spots to transfect the nucleotides. The slides was ever

transfected as soon as one hour after printing, however, they were normally kept for at least 4 hours before cell addition.

Materials (those for cell culture and cell preparation are shown in section 3.4)

- "Gelatin-DNA"-, "lipid-DNA", or "lipid-siRNA"-arrayed slides (see above)
- Effectene Transfection Reagent Kit containing EC buffer, Enhancer and Effectene (Qiagen, Hilden, Germany)
- QuardriPERM plates (Greiner bio-one, Frickenhausen, Germany))
- HybriWell™ Sealing, 40 × 22 × 0.25 mm (#HBW2240, Schleicher & Schuell, Grace Bio-labs)
- Petri Dish, 100 ×15 mm square (Falcon®, Becton Dickinson)
- Human Embryonic Kidney (HEK) 293T cells or HeLa Cells
- Conical tube, 50 ml (Falcon®, Becton Dickinson)
- Vortex Genie 2, VWR
- Cell culture dishes, 100 × 20 mm and 150 × 20 mm (TPP, Switzerland)

3.2.3.1. Cell preparation for reverse transfection

24 hours before the day of transfection, cells were split and plated with a certain amount into new culture dishes. For Hek293T cell line, 10×10^6 cells were plated in 10 ml media into a 100 × 20 mm dish; for HeLa cell line, 8×10^6 cells were plated in 20 ml meida into a 150 × 20 mm dish. Alternatively, 5×10^6 Hek293T cells and 3×10^6 HeLa cells could be cultured for 2 days before transfection. In both cases, cells were still actively growing at a nearly confluent density when harvested. Immediately before reverse-transfection, in a tissue culture hood the cells are harvested following routine protocols. The harvested cells were counted and diluted in a 50 ml conical tube (Falcon) to reach a strict concentration: to cover one printed slide (76 × 26 × 1 mm) in one well of QuadriPerm chamber (Greiner), 3.5×10^6 Hek293T or 1.0×10^6 HeLa cells were aliquoted in 8 ml culture media. The cells were mixed thoroughly before use. If the slides were not ready for transfection, cells could be kept in the tissue culture incubator, however, for no longer than 40 minutes. After storage, cells were always re-suspended by pipeting or inverting the tube for a few times immediately before adding to slides.

3.2.3.2. Reverse transfection of "lipid-DNA" and "lipid-siRNA" slides

The slides printed with "lipid-DNA" and "lipid-siRNA" samples were ready for transfection without additional treatment of transfection reagents. Since this type of slides were normally stored at 4°C, it was very important to let the slides back to room temperature before taking them out of vacuum desiccator. In a tissue culture hood, the slides were put into each well of QuadriPerm plate (Greiner). The cells previously prepared were gently added on top of each slide along with the areas without spots. Without agitation, the QuadriPerm plate was slowly moved into a normal 37°C, 5% CO2 humidified incubator and the cell arrays were cultured for desired hours.

3.2.3.3. Reverse transfection of "gelatin-DNA" slides

The slides printed with "gelatin-DNA" samples have to be treated with transfection reagents before adding the cells. Effectene Transfection Reagent Kit (Qiagen) containing EC buffer, Enhancer and Effectene was used for the transfection of slides printed with Chr21ORF-containing plasmids. The ratio of EC buffer : Enhancer : Effectene was always set at 150 : 16 : 25. In a 1.5 ml micro-centrifuge tube, 16 µl Enhancer was added to 150 µl EC Buffer and incubated for 5 minutes at room temperature. 25 µl Effectene Transfection Reagent was then added and mixed by lightly vortexing for 3-4 seconds (setting 4 on Vortex Genie 2, VWR). The total 190 µl of the transfection reagent mixture was enough to treat one printed slide with the help of HybriWell™ Sealing (HBW2240, Grace Bio-labs). To use HybriWells, follow the steps described below:

1) Peel off the adhesive from the HybriWell and lay the HybriWell in a clean square dish with the adhesive side facing up.
2) Using a 200µl pipette tip to move all the transfection mix (~191µl) to the center of HybriWell, and then slowly put one Gelatin-DNA printed slide on the top of HybriWell and let the transfection reagent evenly distribute over the slide.
   Alternatively, the slide can be firstly attached to HybriWell and the transfection mix can be pipetted through one of the ports of the HybriWell to cover the slide.
3) Incubate the array with the transfection reagent for 20minutes before sucking off the reagents via vacuum or pipetting force.

4) Pull off the HybriWell using a thin tipped forceps or more easily with the fingers, and place the slide into one well of QuardriPERM plate.

The addition of cells and culturing of slides were the same as for "lipid-"slides.

3.3. Transient transfection in 6-well plate

One day before transfection, cells under routine culturing were harvested and a certain amount of cells were counted and seeded into 6-well culture plate (TPP, Switzerland) in which a sterile glass coverslip (20 × 20 mm, Menzel-Glaeser, Germany) was previously laid at the bottom of each well. 0.6×10^6 HEK293T cells or 0.2×10^6 HeLa cells in 2 ml normal culture media were then added on top of each coverslip and cultured under normal growth conditions till the time of transfection. Effectene Transfection and HiperFect Transfection reagents (both from Qiagen) were used for transfection of DNA plasmids and siRNAs, respectively. At the time of detection, the cells on coverslips were subjected to functional assays for protein localization or apoptosis study. The process of functional detection of cells on glass coverslips was not different from the cells growing on transfected cell arrays (see 3.5 and 3.6).

3.3.1. Transfection of DNA plasmids

Effectene Transfection Reagent Kit (Qiagen) was used for transient transfection of DNA plasmids in 6-well plates. One microgram of DNA plasmid was usually used in this study for the transfection in each well. The ratio of EC buffer : Enhancer : Effectene was always set at 150 : 8 : 25 for use, and the transfection was carried out following manufacture handbook.

3.3.2. Transfection of siRNAs

The normal transient transfection of siRNA was carried out in 6-well plates using HiperFect transfection reagent (Qiagen, Inc.). The so-called "fast-forward" transfection protocol (from the manufacture instruction of the transfection reagents) was used to transfect siRNA at a final conentration of 5~10 nM.

3.4. Cell culture

Human HEK293T/17 (ATCC, CRL-11268) and HeLa (ATCC® Number: CCL-2™) cells were routinely cultured in D-MEM medium (Gibco Invitrogen, Karlsruhe,

Germany) supplemented with 10% fetal bovine serum (Biochrom AG, Berlin, Germany), 2mM L-glutamine (Gibco Invitrogen) and 50 units/ml penicillin and 50 µg/ml streptomycin (Gibco, Invitrogen) at 37 °C in a humidified 5% CO_2 incubator. In this media the cells had a doubling time of about 20 hours and were split every 3-4 days to avoid confluency.

3.5. Protein co-localization detection

General Materials

- Paraformaldehyde, formaldehyde (Merck, Darmstadt, Germany)
- Triton X-100 (Sigma)
- Saponin (Carl Roth GmbH., Karlsruhe, Germany)
- Bovine serum albumin (BSA) (PAA Lab.)
- Blocking solution (5.0 % BSA in PBS)
- Antibody dilution solution (0.5% BSA in PBS)
- Mounting reagent-1: Prolong Gold Antifade Reagent (Molecular Probes)
- Mounting reagent-2: Fluoromount-G (Southern Biothechnology)

Specific materials were indicated in the corresponding sections.

3.5.1. Immunofluorescent labeling of His_6-tagged proteins

Antibodies against His_6-epitope were used to label the expressed proteins with a His_6 tag at the N-terminus. In this study, 9 anti-His antibodies produced by different suppliers were compared in parallel (Table 2.2), and Penta•His Alexa Fluor 555 or 488 antibodies from Qiagen gave the most sensitive and specific labeling (see also 4.1.2), and were used throughout the entire study.

After the expression of His_6-tagged recombinant proteins, the cells growing on cell arrays were gently washed once with PBS, followed by a 20-minutes fixation at room temperature with 3.7% paraformaldehyde in the PBS containing 4.0% sucrose. The fixed slides were rinsed for 1-2 minutes in PBS before immediate process of immunofluorescence, or alternatively, the rinsed slides were stored at 4°C in PBS for up to one week before examination.

At the time of examination of anti-His antibodies, the fixed slides were executed following the steps:

1) Permeabilize the slides for 15 minutes in 0.1% Triton X-100 in PBS or 0.5% saponin in PBS, followed by one times rinse.
2) In a humid chamber, block slides for 1 hour with 500 µl of blocking solution (5.0% BSA in PBS) for each.
3) Drain excess blocking solution from the slides, and, without rinse, add 500 µl of anti-His antibody that was freshly diluted in antibody dilution solution (0.5% BSA in PBS) for 2 hours' incubation. Another primary antibody that is not cross-react with anti-His antibody, i.e. it is produced from the host species far different from the host where anti-His antibody originates, were incubated together in this step.
4) Wash slides 3 times of 5 minutes in PBS.

For the slides stained with Penta•His Alexa Fluor 555 or 488 antibodies, directly go to step 6).

For the slides stained with the anti-His antibodies without fluorochrome conjugation, follow the steps below.

5) Re-block slides for 20 minutes with blocking solution before add 500 µl of fluorochrome-conjugated secondary antibody (Table 2.3) that was freshly diluted in antibody dilution solution and incubate for 40 minutes.
6) Wash slides 3 times of 5 minutes in PBS. Let the slides try in the air and mount the slides with Prolong Gold Antifade Reagent following the manufacture's instruction.

The labeled slides were stored in the dark at 4ºC until the examination with fluorescent microscopy or laser scanning. During the entire labeling process, special attention was paid to protect cells from drying out, and the slides were kept in dark to avoid fluorescence bleaching.

3.5.2. Cell organelles counterstaining

A batch of organelle-specific antibodies and/or dyes was screened to label each cellular compartment with the optimization of each staining protocol. The one giving the most sensitive and specific staining was chosen for colocalization study of Chr21 proteins via counterstained with Penta•His Alexa555 or Alexa488 antibodies. The successful or failed antibodies and dyes were listed in Table 3.3, and the methods for

the counterstaining with Penta•His Alexa555 or Alexa488 antibodies were described below.

Table 3.3 Organelle markers used for colocalization study of Chr21 proteins

Organelle	Marker	Counterstaining Method	Supplier
	Successful Markers		
Nucleus	DAPI	1	Sigma
F-Actin	Phalloidin (Rhodamine-conjugated)	2	Molecular Probes
Mitochondria	Prohibitin antibody (rabbit)	3	Abcam
Peroxisome	Catalase antibody (rabbit)	3	Abcam
ER	PDI (mouse)	4	Stressgen
Golgi	Adaptin- γ (mouse)	4	BD Biosciences
Lysosome	LAMP2 (mouse)	4	H4B4, Developmental Studies Hybridoma Bank
Microtubule	α-tubulin (mouse)	4	Sigma
Intermediate filament	Vimentin (mouse)	4	Affinity BioReagents
	Failed Markers		
Golgi	Lectin GS-II		Molecular Probes
ER	Calreticulin antibody (rabbit)		Abcam

3.5.2.1. Counterstaining method 1

The blue fluorescent DAPI stain (Sigma) was used to label the nuclear dsDNA. For each cell array slide, DAPI was always performed after all the other stainings. Right before the last step of PBS washing, the working solution of DAPI (300nM) was diluted from the DAPI-dimethylformamide (DMF) stock solution (prepared following the manufacture's instruction) and was added on cell arrays to incubate the cells for 5 minutes. After three times PBS washing, the slides were mounted as mentioned above.

3.5.2.2. Counterstaining method 2

Phalloidin, a bicyclic peptide, was used to stain F-actin specifically (Wieland and Govindan, 1974). For co-localization of Chr21 proteins with F-actin, the working solution of Rhodamine-Phalloidin (0.5 μg/ml) (Molecular Probes) was prepared together with mouse Penta•His Alexa 488 antibody (2.5 μg/ml) with antibody dilution solution. Following the protocols described in 3.5.1, the cell array slides were fixed, permeabilized and blocked. The incubation of Rhodamine-Phalloidin and

Penta•His Alexa 488 antibody was fulfilled at room temperature for two hours, followed by DAPI staining and final washing and mounting.

3.5.2.3. Counterstaining method 3

Prohibitins are ubiquitous, abundant and evolutionarily strongly conserved proteins located in the inner membrane of mitochondria (Ikonen et al., 1995; Nijtmans et al., 2000). They appear to be very reliable markers of mitochondria in many applications. Catalase is the classical marker for peroxisomes and is the most abundant protein within peroxisomes (Bendayan and Reddy, 1982).

In the counterstaining method 3, cell arrays were fixed with -20°C 100% methanol (can contain 2% formaldehyde) for 10 minutes, followed by 3 times PBS wash. Without permeabilization, the cells were directly performed with blocking solution before antibodies incubation. Anti-prohibitin and anti-catalase antibodies (Abcam) were diluted to their working concentration at 5 μg/ml and 10 μg/ml, respectively, together with Penta•His Alexa 555 or 488 antibody (2.5 μg/ml). Primary antibodies incubation, washing and re-blocking of the cell arrays were executed following the same conditions described in 3.5.1. Staining with the secondary antibodies was performed with Alexa488-conjugated donkey anti-rabbit IgG antibody (~6 μg/ml) (Molecular Probes) or Cy^3-conjugated mouse anti-rabbit IgG antibody (~5 μg/ml) (Jackson ImmunoResearch), depending on which Alexa Fluor of His antibody was used. The conditions of DAPI staining and mounting of the cell arrays were the same as aforementioned.

The His-antibody staining method described in 3.5.1 was not ideal for anti-prohibitin labeling. Different fixatives were found to influence, to a great extent, the staining performance of the prohibitin-antibody. Labeling of prohibitin following formaldehyde-PBS fixation led to high background, whereas the staining in the presence of methanol-based fixative was specific and strong (Fig. 3.4). Methanol fixative, however, was not found to improve the staining performance of the rabbit antibody from Abcam Inc. against calreticulin, an ER specific protein (Fig. 3.4).

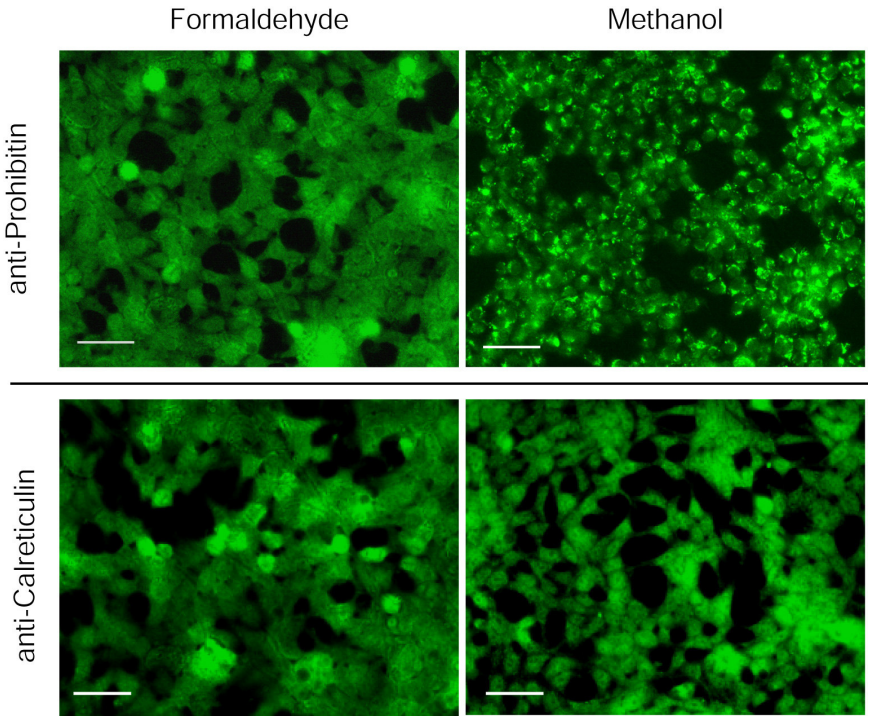

Fig. 3.4 Different fixatives influence the staining performance of anti-prohibitin antibody

Immunofluorescent labeling of prohibitin (a mitochondrial inner membrane protein) and calreticulin (an ER-specific protein), were performed with a rabbit anti-prohibitin antibody (Abcam) and a rabbit anti-calreticulin antibody (Abcam), respectively, together with Alexa488-conjugated donkey anti-rabbit IgG antibody (Molecular Probes). Different fixatives, 3.7% formaldehyde in PBS (left) or 100% methanol (right), were found to improve the staining performance of prohibitin antibody, but had no effect on the performance of calreticulin antibody. In prohibitin staining, labeling with formaldehyde is unspecific with high background, whereas the staining in the presence of methanol-based fixative is specific and strong. Scale bar, 50 μm

3.5.2.4. Counterstaining method 4

PDI (protein disulfide isomerase), Adaptin-γ, LAMP2 (Lysosome-associated membrane glycoprotein 2), α-tubulin, and Vimentin have been known to specifically localized in the ER lumen, the vesicles at Golgi complex, the membrane of lysosomes and endosomes, the microtubules, and the intermediate filaments (Cumming et al., 1984; Franke et al., 1979; Gough and Fambrough, 1997; Noiva and Lennarz, 1992; Robinson, 1990).

Mouse antibodies against these organelle-marker proteins (table 3.3) were found to perform specifically when used alone. The counterstaining of these mouse antibodies with mouse Penta•His Alexa Fluor antibodies, however, had to be performed separately before His-antibody staining to avoid cross-reaction.

Treatment of cell arrays for immunofluorescent labeling with mouse organelle-specific antibodies was fulfilled following the conditions described in 3.5.1, but without the use of anti-His antibody. The working solution of the antibodies were prepared at 4 μg/ml for anti-PDI, 5 μg/ml for anti-Adaptin-γ, 1.5 μg/ml for anti-LAMP2, 8.5 μg/ml for anti-α-tubulin, and 1:30 dilution time for the anti-Vimentin ascites. The incubations were performed for more than 2 hours at room temperature or for overnight at -4°C. The secondary antibody labeling was performed with Alexa488- or Alexa568-conjugated goat anti-mouse IgG antibody (3~6 μg/ml) (Molecular Probes). The excess of the secondary antibodies were then washed thoroughly with PBS for 4 times of 5 minutes. To block the remaining IgG-binding sites on the secondary antibody, 20% normal mouse serum was used as a source of IgG to incubate the cell arrays for 1 hour at room temperature. After draining the excess serum, Penta•His Alexa 555 or Alexa 488 antibody whose fluorescence color is different from the secondary antibody was added to detect the His_6-tagged recombinant proteins. After 2 hours incubation at room temperature, the cell arrays were subjected to the DAPI staining, final washing and mounting.

3.6. Cell apoptosis detection

In this study, several apoptosis assays (Table 3.4) were screened and their staining protocol were optimized for the use on cell arrays. TUNEL, Biotin-conjugated Annexin V and anti-cleavedCaspase3 were found to give the most sensitive and specific staining, whereas the rest of the assays appeared to stain the apoptotic cells weakly or unspecifically.

Table 3.4 Apoptosis assays evaluated on cell arrays

Assays	Detection principle	Supplier
In Situ Cell Death Detection Kit, Fluorescein	DNA fragmentation	Roche
Cleaved caspase-3 (Asp175), Ab (rabbit) Cleaved caspase-7 (Asp198) Ab (rabbit) Cleaved caspase-9 (Asp330) Ab (rabbit) Cleaved PARP (Asp214) Ab (rabbit)	Caspases activation	Cell signaling
FLICA (FITC-DEVD-FMK)	Caspase-3 activation	Biovision
FLICA (FITC-VAD-FMK)	Multiple caspases activation	Biovision
Annexin V-Biotin	Loss of membrane asymmetry	Caltag laboratories
Annexin V-FITC	Loss of membrane asymmetry	BD Pharmingen

3.6.1. TUNEL reaction

Cell arrays were fixed with 3.7% formaldehyde or Paraformaldehyde for 20 minutes at room temperature. After 2 times PBS wash, the permeabilization was performed with 0.1% TritonX-100 in 0.1% sodium citrate for 2 minutes at room temperature. After PBS wash once, appropriate volume of freshly prepared TUNEL reaction mixture (In Situ Cell Death Detection Kit, Fluorescein, Roche) was added on the slides and incubated in a humid atmosphere for 60 minutes at 37°C in the dark. After 3 times PBS wash, the cell arrays were mounted for fluorecent microscopy or scanner analyses.

The fluorescence intensity of Fluorescein-labeled TUNEL reaction, however, was found a little weak and easy to fade, especially during microscopy observation under the higher magnifications. Therefore, in many cases of TUNEL detection in this study, AlexaFluor 488-conjugated anti-Fluorescein/Oregon Green goat antibody (Molecular Probes) was used to amplify the fluorescence signal while still allowing fluorescein-compatible optics. The use of anti-Fluorescein antibody was very similar to the use of a secondary antibody aforementioned. The cells were blocked with 5% BSA in PBS for 60 minutes before incubated with the anti-Fluorescein antibody for 40 minutes at room temperature. If observation of cell nucleus was preferred, DAPI staining was also performed before the final washing step.

In many cases of this study, TUNEL reaction was used together with another immunofluorescence labeling, such as anti-His or anti-Bax staining, rather than used alone. In these cases, the cell arrays were firstly subject to the staining protocol of His or Bax staining using the specific antibody. TUNEL incubation at 37°C was always performed right after the incubation of primary antibody, and before a thorough PBS wash and the incubation of all secondary antibodies.

3.6.2. Cleaved caspases and PARP antibodies

Labeling of cleaved caspase-3, -7, -9 and PARP with the antibodies were performed following the basic immunoflurescence protocol described in 3.5.1. Different dilution times of each antibody were compared, and 1: 50~75 were found optimal for cleaved caspase-3 and PARP antibodies. The staining using cleaved caspase-7 and –9 antibodies were not successful in terms of labeling specificity and intensity.

In this study, cleaved caspase-3 and PARP antibodies were often used with another non-rabbit primary antibody such as mouse anti-His or mouse anti-Bax antibody, in order to reveal the correlations between the activation of caspase-3 or PARP and the overexpression of Chr21 and Bax proteins. In these cases, rabbit caspase-3 or PARP antibody was prepared with either of the mouse antibodies and used to incubate the cell array overnight at 4°C. Alexa488-conjugated donkey anti-rabbit IgG and Alexa568-conjugated goat anti-mouse IgG were used as secondary antibodies, and were mixed together to incubate the cell arrays.

3.6.3. Annexin V

Based on the detection principle (see 1.3.4.), the use of Annexin V requires live, unfixed and nonpermeabilized cells. In this study, it was used before fixation of cell arrays.

At the time of detection, cell arrays were washed once with the PBS containing Ca^{2+} (Gibco, Invitrogen) to remove the culture media, followed by a wash with Annexin V binding buffer (BD Pharmingen). Annexin V working solution was then added to incubate the cells for 20~30 minutes at room temperature. For Annexin V-biotin (Caltag Laboratories), a dilution time with 1:40 was found optimal, whereas for Annexin V-FITC (BD Pharmingen), even the original 10× stock solution was found to stain the apoptotic cells weakly. The Annexin V-biotin-labeled cell arrays were rinsed twice in Annexin V binding buffer before subjected to streptavidin binding. Alexa Flour 594- or 488-conjugated streptavidin (Molecular Probes) was freshly prepared in Annexin V binding buffer to 3~5μg/ml, and used to incubate the cell arrays for 20 minutes at room temperature. After remove the excess streptavidin by washing the slide with Annexin V binding buffer, the apoptotic cells stained by Annexin V were then ready for immediate observation by fluorescent microcopy. Alternatively, for prolonged transportation and storage, the Annexin V-labeled cell arrays were fixed with 4% Paraformaldehyde in PBS for 20 minutes at room temperature. The fixed cell arrays were then mounted for fluorescence examination, or alternatively, were subjected to further immunofluorescent labeling using antibodies against several intracellular proteins, such as Bax, Caspases, and Claudins (see 3.6.5).

3.6.4. FICA

Labeling the apoptotic cells using FLICA (FITC-DEVD-FMK and FITC-VAD-FMK) were performed before cell fixation. At the time of detection, the working solution of FLICA were prepared by diluting the FITC-DEVD-FMK and FITC-VAD-FMK stock with normal culture media at 1:150-fold. Cell arrays were gently rinsed once with fresh culture media that was pre-warmed to 37°C, followed by 1 hour's incubation with FLICA working solution under normal culture condition in the incubator (37°C with 5% CO_2). Unbound FLICAs were removed by rinsing the slides once with culture media and twice with FLICA reaction Wash Buffer from CaspGlow Fluorescein Caspases Staining Kit (BioVision). The cell arrays were then fixed with 1.6% Paraformaldehyde that was freshly prepared in the Wash Buffer. After one time's rinse with the Wash Buffer, the cell arrays were mounted with Fluoromount-G for microscopy examination. Alternatively, the slides were permeabilized with 0.1% TritonX-100 to perform other detection assays such as immuonfluorescent labeling of Bax and PARP proteins. The labeled FLICA signals, however, were found to diminish during severe treatment of the immunofluorescent labeling.

3.6.5. Multiple apoptosis detection (Triple death assay)

Three apoptosis assays—Annexin V, cleaved caspase-3 and TUNEL reaction—were applied together on one cell array slide in order to collect the death signals from different apoptosis stages in a single test.

The cell arrays were always labeled with Annexin V prior to cell fixation. After fixation, cell array slide was stored in Annexin V binding buffer at 4°C until multiple detection. The multiplex detection was performed following the protocol listed below.

1) Permeabilize the cell arrays for 10 minutes at room temperature with 0.1% TritonX-100 in PBS.

NOTE: The permeabilization solution used for TUNEL reaction (0.1% TritonX-100 in 0.1% sodium citrate, from the manufacture's intruction) should be avoided as the AnnexinV staining faded in this solution.

2) Remove the excess permeabilization solution and rinse the cells with AnnexinV binding buffer once.
3) Block the cell array with 5% BSA in PBS for 1 hour at room temperature in a humid environment.

4) Incubate the cell array with primary antibody, i.e. rabbit cleaved caspase-3 in this case, overnight at 4°C or for at least 2 hours at room temperature.
5) Drain the excess primary antibody solution and, without wash, perform TUNEL reaction for 1 hour at 37°C.
6) Wash the slide thoroughly with PBS for 4 times of 5 minutes.
7) Re-block the cell array with 5% BSA in PBS for 20 minutes at room temperature.
8) Incubate the cell array with secondary antibodies for 40~60 minutes at room temperature.

NOTE: The secondary antibodies solution could include an anti-FITC antibody and an anti-Rabbit antibody without cross-activity. Depending on the experiment purpose, if the signals from caspase-3 and TUNEL needed to be combined, AlexaFluor488-conjugated goat anti-rabbit and AlexaFluor488-conjugated donkey anti-FITC secondary antibodies were used; if not, AlexaFluor555-conjugated anti-Rabbit antibody was used rather than AlexaFluor488-conjugation.

9) Label the cell nuclei with DAPI working solution for 5 minutes.
10) Wash the slides 3 times of 5 minutes before mounting with Fluoromount-G.

The mounted cell arrays were ready for cell death identification with fluorescence microscopy and scanner.

In order to monitor the cell death induced by overexpression of certain Chr21 proteins and Bax protein, the multiple apoptosis assays were combined with mouse anti-His or with anti-Bax antibody staining. Following the protocol of apoptosis assays, mouse Penta·His Alexa555 antibody or unconjugated mouse Bax antibody was incubated together with the rabbit caspase-3 antibody. In this case, Alexa488-, not Alexa594-, conjugated Streptavidin was previously used and the solution of secondary antibodies included Alexa488-conjugated goat anti-FITC antibody, Alexa488-conjugated donkey anti-rabbit antibody, and Alexa568-conjugated Goat anti-mouse antibody. As a result, all apoptosis assays were labeled with green fluorescence, while His-tagged Chr21 proteins and Bax protein were labeled with red fluorescence.

3.7. Immunofluorescent detection of Bax, lamin A/C and hnRNP-A3

Mouse anti-Bax antibody (clone 3, BD Biosciences) was used to evaluate the expression level of exogenous Bax protein. The working solution of the antibody was

freshly prepared at 1.6 μg/ml before use. For Hek293T and HeLa cell lines, the endogenous Bax proteins were barely detected in the cells not undergoing apoptosis, whereas the overexpressed exogenous Bax proteins were intensively labeled. Counterstaining of Bax with different apoptosis assays were performed, and the optimal protocols were described above in this section.

Mouse anti-human Lamin A/C antibody (clone JoL2, CHEMICON international) and rabbit anti- hnRNP-A3 antibody (a kind gift from Karl Skriner, Charite medical center, Germany) were used to evaluate the silence efficiencies of their specific siRNAs. The working solution of the mouse and rabbit antibodies was prepared with the dilution times at 1:30 and 1:500, respectively, and was used to incubate the cell arrays overnight at 4°C or for at least 2 hours at room temperature. Alexa568-conjugated Goat anti-Mouse antibody and Alexa488-conjugated donkey anti-rabbit antibody were used as secondary antibodies to label the endogenous lamin A/C and hnRNP-A3 with different fluorescence. Like other labeling methods, cell nuclei on the arrays were stained with DAPI before final washing and mounting.

3.8. Image acquisition and analyses

Each labeled cell array was firstly scanned via using a BioCCD scanning system (Applied Biosystems, Darmstadt, Germany). The excitation and emission filter sets for the fluorochromes used in this study were listed in Table 3.5. The scanned images were analyzed using GenePix 6.0 (Axon Laboratory) software. To evaluate the siRNA-induced gene silencing and apoptosis, each cell cluster that was transfected by one siRNA spot was treated as one dataset and the quantification was performed by mean intensity of the transfected cell cluster after background correction. For cell morphology analysis and protein subcellular localization, ImagerZ1 microscope (Zeiss, Jena, Germany) and LSM510 confocal system (Zeiss) were used, together with Axiovision 4.0 and LSM510 software (Zeiss).

Table 3.5 The filter sets used for image acquisition with BioCCD scanning system

Fluorescence	Exitation Filter	Emission Filter
FITC, EGP, AlexaFluor 488	470/30	510/20
Cy^3, AlexaFluor 568, AlexaFluor 555	565/20	596/14
Alexa Fluor 594	594/14	622/14