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Towards a cellular multi-parameter analysis platform: Fluorescence *in situ* hybridization (FISH) on microhole-array chips

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***Abstract*—Highly-sensitive analysis systems based on cellular multi-parameter are needed in the diagnostics. Therefore we improved our previously developed chip platform for another additional analysis method, the fluorescence *in situ* hybridization. Fluorescence *in situ* hybridization (FISH) is a technique used in the diagnostics to determine the localization and the presence or absence of specific DNA sequence. To improve this labor- and cost-intensive method, we reduced the assay consumption by a factor of 5 compared to the standard protocol. Microhole chips were used for making the cells well addressable. The chips were fabricated by semiconductor technology on the basis of a Silicon wafer with a thin deposited silicon nitride layer (Si_3N_4). Human retina pigment epithelia (ARPE-19) cells were arrayed on 5-µm holes of a 35x35 microhole-array by a gently negative differential pressure of around 5 mbar. After 3 hours of incubation the cells were attached to the chip and the FISH protocol was applied to the positioned cells. A LabView software was developed to simplify the analysis. The software automatically counts the number of dots (positive labeled chromosome regions) as well as the distance between adjacent dots. Our developed platform reduces the assay consumption and the labor time. Furthermore, during the 3 hours of incubation *non-invasive* or *minimal-invasive* methods like Raman- and impedance-spectroscopy can be applied.**

I. Introduction

THE early detection of tumor cells or cancer-promoting cellular mutations is important in the diagnostics, prognosis and for the choice of therapy. In the early stages the relationship between mutated/cancer and healthy cells is very low and the detection method has to be very sensitive. To give an example: 1 out of 10^3-10^7 nucleated cells in blood is a cancer cell [1]. To increase the sensitivity and to increase the detection rate of mutated cells multi-parameter analysis systems are needed.

For the cellular analysis on genetic level, karyotyping and fluorescence *in situ* hybridization (FISH) as cytogenetic test as well as the polymerase-chain reaction (PCR) as molecular genetic test are used. Some cryptic information at gene level gets lost in the PCR method, like translocation and rearrangement, when averaging over high number of cells [2]. The advantage of FISH against karyotyping are that it is faster and larger number of cells can be treated simultaneously [3].

The fluorescence *in situ* hybridization (FISH) is a sensitive diagnostic tool, which can sense gene alterations like amplifications, translocations in the DNA [4]. A well known example for changes in the gene is trisomy 21, where the chromosome 21 or parts of it occurs three times in the DNA. Because of the complexity, the high expenditure and the high expenses (approximately $90 per test slide [3]), FISH is nowadays still infrequently used in clinical situations [5]. Therefore optimizations have to be found to limit the FISH probe reagent consumption and to reduce the working time.

The development of chip-based systems is temporarily focusing on the miniaturization on assays like PCR or DNA microarrays [6]. In the past there have only been few reports on FISH on chip [3, 5] and most of this research is focusing on the reduction of assay consumption and the amount of labor. But none of these reports is dealing with the combination of different diagnostic methods.

In earlier reports we showed the multi-parameter feasibility of our developed microhole-chip based system at two examples and combined it with impedance-spectroscopy [7] as well as Raman-spectroscopy [8]. Cells were entrapped on microholes by a gently suction pressure. 35x35 microhole-array chips with a 1 µm thin Si_3N_4 membrane are used for a high quality optical characterization. The chip membrane sizes are easily scalable and allows a characterization of millions of cells on one chip.

In this article we expand our system for the FISH method and show a reduction of FISH reagent by a factor of 5. We developed also an image processing algorithm, which counts the signal dots and also calculates the distance between adjacent dots.

II. Materials and Methods

A. Fabrication of microhole-array chip

The fabrication of the chip was done by semiconductor technology [9]. A 4 inch two-sided polished (100) silicon wafer was used with a thickness of 300 µm. A 1 µm thick Si_3N_4 layer was deposited on both sides by plasma-enhanced chemical vapor deposition (PECVD). After photolithography

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and reactive ion etching (RIE) holes were opened on the wafer front side whereas the silicon nitride layer on the back side of the wafer was opened in the region above the array by the same process. This opening was used as an etch mask for the anisotropic etching of the silicon wafer in 25 % KOH at 80 °C. The resulted etching walls lie on the (111) crystal plane. The etch angle from (100) to the (111)-plane is 54,7°. A 35x35 hole-array was used for the experiment with a pitch of 40 µm. The Si_3N_4 membrane has a square shape with a length of 2.13 mm. The outer dimension of the chip is 4.4x4.4 mm^2.

B. *Cell culturing*

In this work retina pigment epithelial cells (ARPE-19) were used. The cells were cultivated in an incubator (Heraeus BBD6220, Hanau, Germany) at a temperature, CO_2 and humidity of 37 °C, 5 % and 95 %, respectively. For the experiments the cells were trypsinised, centrifuged and resuspended in fresh culture medium and put into a 15 ml falcon tube. The culture medium consisted of DMEM/F12 (Invitrogen/GIBCO, Karlsruhe, Germany), 10 % (v/v) fetal calf serum (FCS) "Gold" (PAA Laboratories GmbH, Cölbe, Germany), 100 units/ml penicillin (Invitrogen/GIBCO) and 100 µg/ml streptomycin (Invitrogen/GIBCO).

C. *Microfluidic and cell positioning*

A fluidic adapter was used for connecting the chip to the fluidic. Macrolon, which is autoclavable and resists 70 % ethanol for sterilization, was used as adapter material. The adapter has one fluidic connection (inlet/outlet). Two sealing rings were used to close the chip with the adapter tightly. The adapter is shown in fig. 1. The negative pressure was generated by a venturi injector (Festo, Esslingen-Berkheim, Germany) and was monitored by a pressure sensor (SensorTechnics, Puchheim, Germany). The input positive pressure to the venturi injector was controlled by a fine regulating valve (BelloFram, Newell, USA).

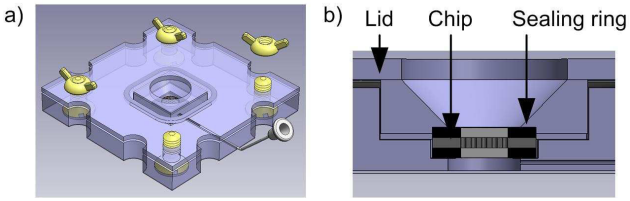

Fig. 1. Fluidic adapter for the connection of the fluidics to the chip. a) the complete adapter with the inlet and the lid closed by screws. b) Enlarged area of the chip sandwiched between two sealing rings.

For the chip/adapter adaption the channel under the chip was filled air-bubble free with cell culture medium. Afterwards the space under the chip was tightly closed by pressing the chip on the sealing ring. The differential suction pressure of around 5 mbar was applied to the chip system and the cell suspension was added. The positioning process was monitored by a microscope (Olympus IX81, Hamburg, Germany). Cells which are not arranged regularly were washed away by using a standard pipette. For realizing cell culture condition an air conditioning unit (Evotec Technologies, Düsseldorf, Germany) around the microscope was used during the positioning and holding of the cells on the chip. The temperature, humidity and CO_2 were adjusted to 37 °C, 80 % and 5 % respectively. Further the spreading and adhesion of the cells to the chip was monitored by the microscope. To reduce the cells movement during the spreading and attaching process the pressure was smoothly increased up to around 10 mbar. After incubation for nearly 3 hours most of the cells were attached to the chip membrane surface and the chip was ready for the FISH procedure.

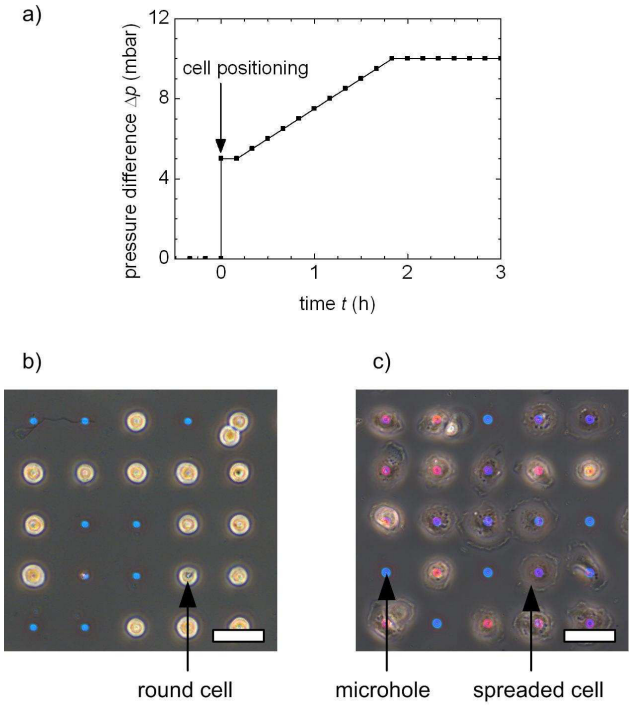

Fig. 2. Positioning of the Arpe-19 cells on the array (bar = 40µm). a) Pressure distribution during the positioning and holding of the cells on the chip. At time $t = 0$ h the cells were entrapped on the holes. After around 20 min the suction pressure was linearly increased to reduce the cells movement on the holes. Phase contrast microscope images: b) round cells directly after positioning, c) attached cells to the chip membrane (different chip area as in 2b)).

D. *Fluorescence in situ hybridization (FISH)*

For the FISH the chips were taken out of the adapter. Several chips (up to five) were treated in parallel. Based on the cytology FISH Accessory Kit from Dako (Dako, Bonn, Germany) the protocol [10] was improved for smaller assay volumes, for labor reduction (without pre-treatment with pepsin) and for the chip handling. Petri dishes (Ø 35 mm) were used for the liquid handling and the chips were put into the solutions by normal tweezers. To get a better solution circulation the petri dishes were rotated by hand.

As mentioned in the protocol the cells were fixed by 3.7 % formaldehyde (FA), washed in wash buffer (WB) and dehydrogenized in a stepwise increased ethanol dilution series (70 %, 85 % and 96 %). The cells on the chip were co-denatured by 2 µl of the FISH probe mix (instead of 10 µl recommended by DAKO FISH protocol). Afterwards the

chip was put onto an objective slide and closed by a cover slip. Cover slip sealant (Marabu, Tamm, Germany) was used to fix the sandwich of the object slide, the chip, and the cover slip. After 14-20 hours of hybridization the sealant was removed and the chips were again washed in wash buffer and stringency buffer (SSC) to remove unspecific bindings. Another dehydrogenizing step was used in the same ethanol dilution series. After drying the chips they were sandwiched between an object slide and a coverslip with mounting media including DAPI (Vecta Lab, Burlingame, USA).

The used EGFR/CEN-7 FISH probe mix (Dako, Bonn, Germany) detects the EGFR (epidermal growth factor receptor) gene located on chromosome 7p11.2. As reference this probe uses the chromosome 7 centomere region.

The FISH results were analyzed by a microscope (CarlZeiss Axio Observer.Z1, Jena, Germany). A 63x oil-immersion-objective (Plan-Apochromat 63x/1.4 Oil M27) was used. The excitation/emission wavelength and illumination times for DAPI, FITC and Texas Red are shown in table 1.

TABLE 1
EXCITATION AND EMMISION WAVELENGTHS AS WELL AS THE ILLUMINATION TIMES FOR THE FISH ANALYSIS ON CHIP. BP STANDS FOR BANDPASS.

	Excitation–Emission	Time (s)
DAPI	BP G365 – BP 445/50	~ 0.03
FITC	BP 470/40 – BP 525/50	~ 0.5
Texas Red	BP 560/40 – BP 630/40	~ 0.5

E. *Software*

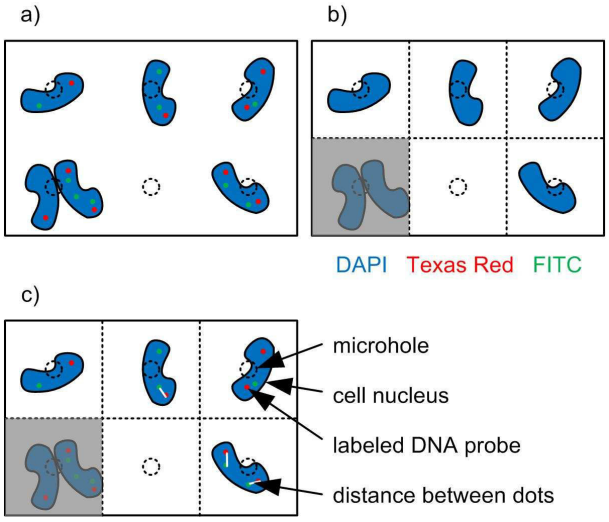

Fig. 3 Software protocol for the FISH analysis on chip. a) Possibility of the arrangement with the different fluorescent labeling. b) Software is using the DAPI-stained cell nuclei for the subdivision of the array. c) Stained DNA probes are used for counting and for analysing the distance between adjacent probs.

The developed algorithm is working on the equally distributed holes. It divides the array into a matrix, where the hole is in the centre of each field. The fluorescent image of the DAPI stained nuclei was taken for the matrix field/cell calibration. Fields with more than one positioned cell ($N > 1$) or cells with touching nuclei ($N = 1$, but nuclei touching each other) were cancelled for the analysis. Therefore only matrix fields with N = 0 and N = 1 but no cell agglomerations were determined. The fluorescent images of the FISH probe mix (FITC/Texas Red) were used for analyzing the number of spots in one matrix field and for analyzing the distance between red and green spots. This procedure is shown in fig. 3. For the distance calculation only the directly adjacent FITC/Texas Red labeled chromosome regions as shown in fig. 3c) are used.

III. RESULTS

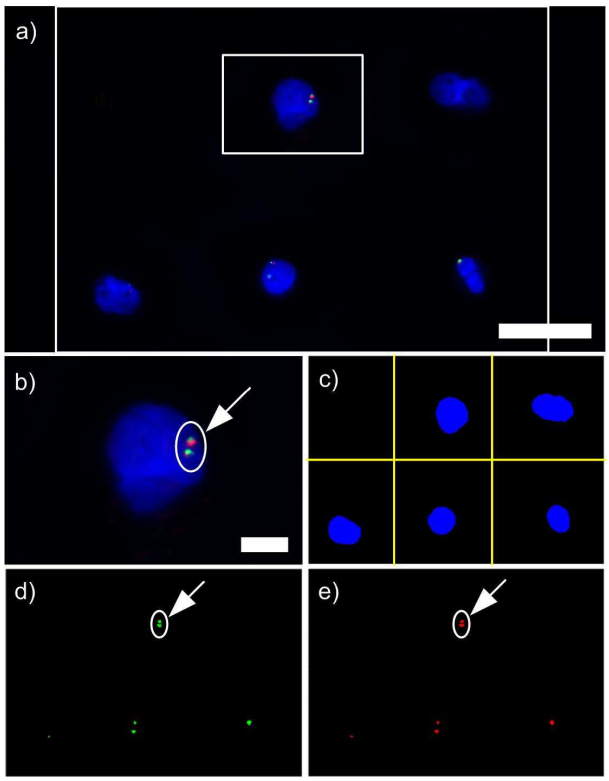

Fig. 4 Flourescent microscope image and binary images of the image processing. a) Overlayed fluorescent images of the DAPI stained nucleus and the Texas Red and FITC labeled chromosome regions (bar = 20 µm). b) Magnified view of the (1x2) cell from 4a). The arrow shows the stained chromosome region. Two red dots and two green dots are visible (bar = 5 µm). Binary image from the software c) DAPI stained cell nucleus with the software developed grid, d) FITC labeled centomeric region of chromosome 7 (arrow shows two signal dots from 4b), e) Texas Red labeled EGFR (epidermal growth factor receptor) gene (arrow shows two signal dots from 4b).

Arpe-19 cells were positioned *non-invasively* on the microholes by a pressure of 5 mbar. The pressure chute shown in fig. 2a) is necessary to keep the cells for a longer time on the holes during the adhesion process. Fig. 2c) shows that the cells are well arrayed on the microhole-array.

For the distances of the labeled chromosome regions in fig. 4a) the values are given in table 2. The distance is calculated only for neighboring FITC/TR pairs. In this case the distances are in the range of 8.20 ± 0.38 pixels (excluded field 4), which is 0.66 ± 0.03 µm (picture size/resolution).

TABLE 2
DISTANCES BETWEEN ADJACENT FITC AND TEXAS RED (TR) CHROMOSOME REGIONS CALCULATED BY IMAGE PROCESSING

Matrixfield	FITC/TR	Distance (pixel)
1	0	-
2	2/2	8.20; 7.88
3	0	-
4	1/1	17.29
5	2/2	7.77; 8.56
6	1/1	8.60

IV. DISCUSSION

The combination of microhole-array chips with fluorescence *in situ* hybridization (FISH) and image processing is promising for an automated FISH analysis with reduced FISH probe mix consumption. Arpe-19 cells were entrapped by a negative pressure of around 5 mbar. In previous studies we showed that such a suction pressure did not increase the number of dead cells [11]. Cells lying between the holes were flushed away by a standard pipette. Therefore no special cell concentration is necessary. The pressure was increased linearly to prevent cells from moving away from the holes. After approximately 3 hours all entrapped cells were spread and attached to the chip membrane surface. For performing the FISH the chip was taken out of the positioning fluidic adapter. By using the chip as cell-carrier the consumption of the FISH reagent is reduced by a factor of five. Compared to literature [3], this value should be further reduced by building up a complete microfluidic system around the chip. Our image processing software is working on the array and uses the DAPI stained cell nuclei for dividing the array in a matrix field. If more than one stained cell nucleus is lying in a field, this field is cancelled, because this can be resulted from a wrong cell arrangement or a cell division. During the cell division mitosis and cytokinesis form two diploid cells, which can lead to misinterpretations in the FISH analysis (only single spots occur).

Criterions in the FISH analysis are gene amplifications or translocations [3], therefore the image processing counts the number of FITC and Texas Red labeled chromosome regions in the cell nucleus and calculates the distance between neighboring signal dots. The distances in table 2 are in the range of 8.20 ± 0.38 pixels (excluded matrix field 4). The distance calculated from the control (analysis of 20 cells on an object slide treated with FISH) is 8.70 ± 2.97 pixels. The high standard deviation might be caused by the signal dots, which are in different layers. Also the calculated distance in matrix field 4 might be based on this effect, i.e. the other labeled genes are located outside the plane of focus and are therefore not clearly visible in the microscope image. Therefore our developed chip system is able to detect gene amplifications and to calculate the distance between neighboring labeled genes for the detection of gene translocations.

V. CONCLUSION

A FISH-on-chip combined with a software algorithm is introduced. Chips with a 35x35 microhole array were used, but the chip sizes can be easily scaled up to allow the FISH analysis of millions of cells on one chip. A DAPI staining is not necessary any more, because matrix fields with more than one cell can be identified during the cell spreading process by phase contrast microscope image.

The introduced chip system platform is suitable for a multi-parameter analysis of single well arranged cells. With impedance spectroscopy and Raman spectroscopy as minimal-invasive methods as well as FISH as strong invasive method on chip, cells can be characterized by its compos-ition, due to its morphology and also on a molecular and genetic level. Therefore such a combination in one system generates a more reliable diagnosis than only one method alone.

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