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Reviews

Microdevice in Cellular Pathology: Microfluidic Platforms for Fluorescence *in situ* Hybridization and Analysis of Circulating Tumor Cells

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Microfluidic devices enable the miniaturization, integration, automation, and parallelization of chemical and biochemical processes. This new technology also provides opportunity for expansion in the field of cellular pathology. Fluorescence *in situ* hybridization (FISH) is a well-known gene-based method to image genetic abnormalities. Development of a FISH microfluidic platform has offered the possibility of automation with significant time and cost reductions, which overcomes many drawbacks of the current protocols. Microfluidic devices are also powerful tools for single-cell analysis. Capturing the circulating tumor cells (CTCs) from blood samples is one of the most promising approaches to enable the early diagnosis of cancer. The microfluidic devices are also useful to isolate rare CTCs at high efficiency and purity. In this review, I outline recent FISH and CTC analyses using microfluidic devices, and describe their applications for the cellular diagnosis of cancers.

Keywords Microfluidics, fluorescence *in situ* hybridization, DNA, circulating tumor cells, pathology

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1 Introduction

Cellular pathology techniques have progressed in recent years. Conventionally, the pathologist observes tissues with a microscope and determines whether or not the hematoxylin and eosin (H&E)-stained tissues contain cancer cells based on their morphology. The H&E stain is the traditional and most widely used staining method in medical diagnoses.[1] Recent advances in genetic analysis methods have made it possible to identify the genetic basis of human diseases, and have opened the door to individualized prevention strategies and early detection and treatment. Therefore, combinations of the traditional morphological diagnosis method and newer genetic methodologies are strongly welcomed.

Fluorescence *in situ* hybridization (FISH) is a well-known gene-based method used to image genetic abnormalities. FISH is a microscopic technique in which specific DNA sequences tagged with fluorophores are used to detect target genes and identify their localization within a cell. FISH was developed in the early 1980s[2] and has since been improved continuously.[3] The FISH probes can only detect genes with a high degree of homology, and can visualize specific cytogenetic abnormalities such as copy number aberrations, gene mutations, and gene fusions.[4] This advanced molecular pathology technique has enabled better diagnoses of diseases, leading to more tailored therapeutic regimens.

Analytes have become more multifaceted. In general, FISH is

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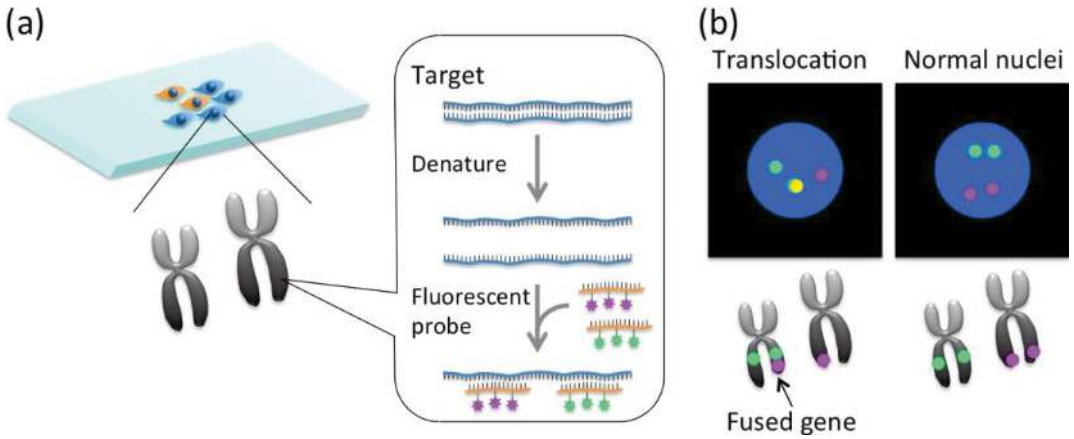

Fig. 1 Principle of fluorescence *in situ* hybridization (FISH).
(a) Overall procedure of FISH. Cells fixed on a slide are pretreated by enzymatic digestion to improve probe penetration. Next, the target sequences on chromosomes are denatured by heat or chemicals. The fluorescent probes specifically hybridize to their complementary sequences on the chromosome. The hybrids formed between the probes and their chromosomal targets can be detected using a fluorescent microscope. (b) Detection of chromosomal abnormalities in interphase nuclei. Two pairs of signals (*e.g.*, two green and two red signals) were observed in normal cells. A fusion gene originating from translocation produced a yellow spot signal that was detected as a colocalized fluorescent signal.

performed on a tissue section isolated from a lesion site. Recently, a new biomarker, circulating tumor cells (CTCs) in the blood,[5] has attracted attention owing to its potential for early cancer diagnosis. CTCs are thought to leave the primary tumor and circulate in the bloodstream, and are thus considered to be precursors of metastasis. Therefore, capturing the CTCs from blood samples is one of the most promising approaches to enable the early diagnosis of cancer.

Despite the many innovations and technological advances made in cellular pathology, use of a glass slide is still a vital part of the FISH process, which has undergone little change. However, the new technology of microfluidic devices provides an opportunity to progress beyond the glass slide. Microfluidic systems enable the miniaturization, integration, automation, and parallelization of chemical and biochemical processes. Microchip electrophoresis has demonstrated several advantages in speed, flexibility, portability, and sample and reagent requirements over standard capillary electrophoresis.[6] There are also several advantages of microchip-based experiments using cultured cells that are difficult or impossible to obtain with cell culture in traditional polystyrene dishes,[7] including the ability of mimicking the cell's natural microenvironment more closely in the continuous perfusion culture, to perform experiments with low numbers of cells or with single cells, parallelization, and on-chip analysis.

In this review, the potential of microfluidic technologies is summarized, which are expected to bring about sweeping changes in the field of cellular pathology. The use of microfluidic technology greatly reduces reaction volumes and corresponding reagent and handling costs. Microfluidics facilitate more rapid analyses as compared to the conventional method and automation of all processes. This review focuses specifically on FISH and CTC analyses in a microdevice, and the feasibility of FISH-based cellular diagnosis of cancers and handling of single cells.

2 Conventional FISH Procedures

FISH is essentially based on the same principle as a Southern blot analysis, a cytogenetic equivalent that exploits the ability of single-stranded DNA to anneal to complementary DNA (Fig. 1). This technique is performed on cells that are chemically fixed to a slide followed by denaturation of DNA and hybridization of the denatured single-stranded DNA inside the cells with fluorescent probes (labeled oligonucleotides or DNA fragments) overnight. Formalin-fixed paraffin-embedded material is the most readily available source of diseased tissue, including tumors. Paraffin-embedded tissue slides require a pretreatment procedure before hybridization, and the samples are analyzed by fluorescence microscopy. Conventional FISH analysis is a time-consuming (takes 2 – 3 days to complete), labor-intensive, and expensive (~$90 per slide[8]) technique.

3 Integrated FISH on a Microfluidic Platform

Microfluidic platforms offer many advantages over conventional diagnosis methods, such as their low cost, ease of use, high portability, and disposability. One of the advantages of miniaturization is to reduce the reagent cost of conventional FISH analysis. Fluorescently labeled DNA probes are the most expensive reagent required for a FISH assay. Microfluidics can reduce the probe volume to several microliters (Table 1). This section summarizes the microfluidic device for FISH.

3·1 Samples and target sequences

A brief summary of applications of a microfluidic device for FISH is provided in Table 1. Most of these reports have only been proof-of-concept studies. In several of these reports, cancer cell lines were used and oncogenes were analyzed, although bacteria analysis was also conducted.[9] Although formalin-fixed paraffin-embedded material is the most readily available source of diseased tissue, there was only one report that involved mouse tissue sections.[10] This is because

Table 1 Microfluidic FISH applications

	Device material	Probe solution volume/μL	Analysis time	Sample	Target sequence	Ref.
1	Glass and polyacrylamide	80	2.5 h	Bacteria	16S rRNA	9
2	PDMS	100 (chamber size)	3 h	Mouse brain tissue section	miRNA	10
3	Cyclo olefin	5	66 min	Human epithelial cell lines: SKBR-3, G-401.	*ERBB2, SE17, HER2*	12
4	APTES- or PEG-coated glass	10	Overnight	Nonadherent cell line: K562	Chromosome 3	13
5	PDMS and TiO_2-modified glass	0.3	Overnight	Tumor cells, bone marrow from normal donor	*P53/ATM, D13S319, 13q14.3, IgH, IGH/CCND1, CBFB, AML1/ETO, BCR-ABL*	14
6	Glass and PDMS	1.0	1 h	Cell lines: RAJI, KMS12-BM, KMS18	*IGH/FGER3*	15
7	Glass and PDMS	0.5 – 1.0	93 min	Peripheral blood mononuclear cells; RPMI 8226	X and Y chromosomes	19
8	PDMS	5	3.0 h	Human glioblastoma cell line MV4-11; whole blood samples	*MLL* gene	20

reproducibility of the FISH analysis of a tissue section is generally poor, since its physical properties (*e.g.*, hardness or permeability) are uneven. Another reason is that RNA is not well preserved in formalin-fixed tissue. Therefore, a new stable fixation method was recently developed,[11] which should increase the number of reports involving tissue sections in the future.

3·2 Materials

The most frequently used material for a microfluidic device for FISH is polydimethylsiloxane (PDMS) or glass. PDMS is often chosen because of its ease of fabrication and optically transparent properties; however, it is not suitable for low-cost mass production of the device. Cyclo-olefin polymer devices were suggested to be cheaper than PDMS chips.[12] Cyclo-olefin polymer is resistant to harsh solvents and temperatures, and enables high-resolution and high-throughput routine mass production by hot embossing or injection molding, which could achieve the production of fully disposable devices. Furthermore, the cyclo-olefin devices present excellent optical quality in both the visible and UV range, allowing for high-quality fluorescence imaging.

There have also been reports on the surface modification and fabrication of microstructures in a microchannel. Liu *et al.*[13] reported modification of a glass surface with (3-aminopropyl) triethoxysilane (APTES) and polyethylene glycol (PEG)-silane for fabrication of a cell array. To promote cell attachment, Zanardi *et al.*[14] performed surface coating with TiO_2 nanoparticles. Fabrication of a filter structure with polyacrylamide in a microchannel was also reported.[9]

3·3 Operation

In the general FISH procedure, a labeled probe hybridizes to its target mRNA or DNA fragment within a cell overnight, because the reaction depends on diffusion-limited hybridization. There is potential to enhance hybridization by mechanical mixing in the microdevice; *i.e.*, integration of FISH into a microdevice achieves reduction in hybridization time.

Sieben *et al.*[15] reported the first microchip-based FISH protocol. The on-chip implementation of FISH allowed for several chromosomal abnormalities associated with multiple myeloma to be detected with a 10-fold higher throughput and 1/10th the reagent consumption of the traditional slide-based method. In addition, the microchip-based method enhanced probe hybridization by using a circulation microchannel with mechanical pumping or straight channels with electrokinetic pumping.

Soe *et al.*[10] precisely analyzed the hybridization efficiency under flow as well as static conditions in a sample-fixed microchannel. They used microscope slides with spotted DNA microarrays inside the microchannel instead of a tissue section. This method not only reduced dispersion of the hybridization signal intensity but also improved hybridization efficiency by using flow-based incubation. Tissue section analysis was performed by FISH using horseradish peroxidase-labeled oligodeoxynucleotides and tyramide signal amplification. Flow-based hybridizations resulted in a significant increase of the specific signal intensity compared to static conditions. However, the reaction efficiency of the antibody was worse in the microdevice compared to that obtained with the conventional glass slide technique. This was considered to be due to reduction in the effective antibody concentration available for interaction with the label on the probe in the device. Therefore, when using an antibody, surface modification might be necessary to minimize nonspecific adsorption.

Furthermore, a microdevice optimized for a metaphase FISH protocol was reported.[16-18] To obtain a clearly visible image, metaphase chromosomes were spread and fixed on the slide in a manner to reduce the overlap of chromosomes. This procedure involved treatment of cells with a hypotonic salt solution, fixation of the suspended cell pellet, and dropping of the cells onto glass slides. Svendsen *et al.*[16-18] introduced a novel splashing device for preparing metaphase spreads on a microscope glass slide. They reported the application of a semi-closed microfluidic chip to fast evaporation of the fixative solution. They further investigated the possibility of obtaining metaphase chromosome spreads in a semi-closed device for chromosome analysis by banding or FISH. They tested the spreading capability in the device with a glass and cyclic olefin copolymer bottom that was chemically modified to have long-term wettability. The usability of the prepared spreads was tested by FISH performed in the microfluidic device.

Analysis of not only human cells but also bacteria has also been reported using flow-based FISH. Liu *et al.*[9] described an integrated microfluidic device capable of performing 16S rRNA

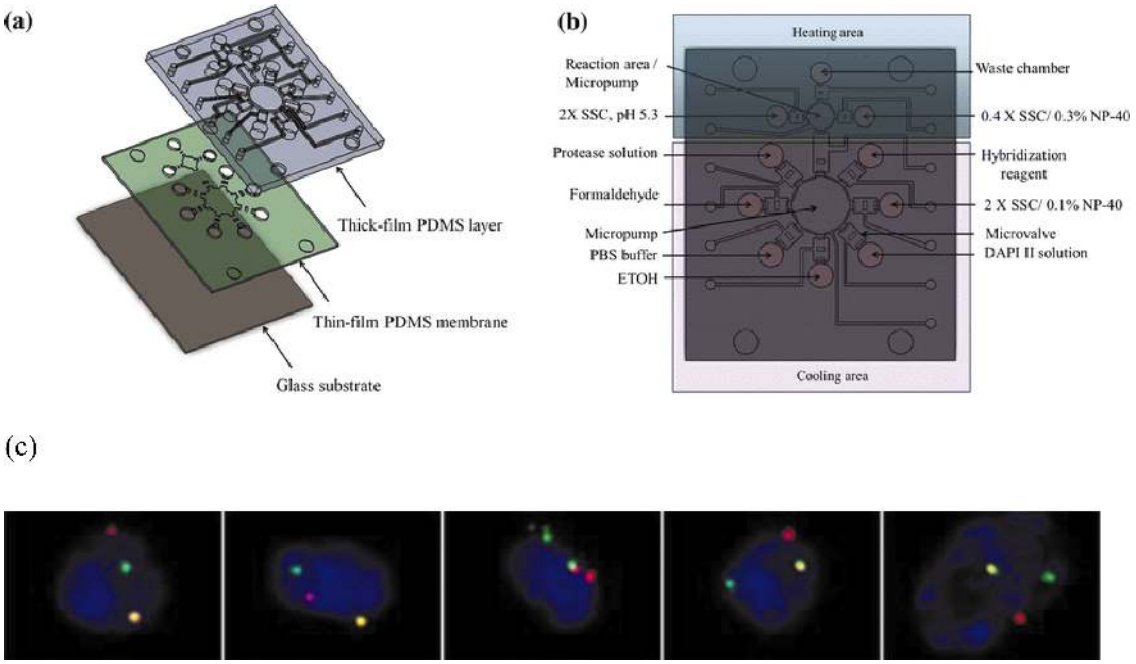

Fig. 2 Integrated fluorescent *in situ* hybridization (FISH) microfluidic platform with a micropump, micromixers, and microvalves. (a) An expanded view of the microfluidic FISH chip consisting of two polydimethylsiloxane (PDMS) layers and one glass plate. (b) A schematic illustration of the integrated FISH chip consisting of a microfluidic control module and a temperature control module. (c) Experimental results demonstrating that MV4-11 cells harbored an 11q23 translocation. Fluorescent images were obtained after the automated FISH protocol was performed on the integrated microfluidic chip. The MV4-11 leukemia cell line served as a positive control for the presence of *MLL* translocations. Each cell has two signals separated into the 5′ (green) probe and the 3′ (orange) probe, presumably because of a translocation. Reprinted with permission from Ref. 20.

FISH followed by flow cytometric detection for identifying wild bacteria. The device seamlessly integrated two components: a hybridization chamber where cells and probes were electrophoretically loaded, incubated, and washed; and a downstream cross structure for electrokinetically focusing cells into a single-file flow for the flow cytometry analysis. Use of this method allowed for 1/10th the sample consumption (from 100 cells) compared to that of a conventional flow cytometer.

Moreover, the microdevice has the advantage of easy automation. The automation systems were equipped with all necessary devices, including a heater, pump, and so on.[19-21] Tai *et al.*[20] developed an integrated FISH chip featuring two temperature control modules, a heating and cooling area, and a suction-type micropump using a PDMS thin film that reduced the vertical height of the reaction chamber and provided gentle mixing, which is crucial for efficient DNA hybridization. The DNA target could be efficiently moved vertically from the top of the channel to the bottom by controlling the PDMS membrane (Fig. 2). The technique yielded substantial reductions in the consumption of samples, reagents, and time (3 h). The developed microfluidic system successfully provided superior performance in probing chromosomal abnormalities in peripheral blood mononuclear cells.

The automation system facilitated easy operation for analysis with multiple reaction steps. Sato *et al.*[21] developed a novel, simplified, automated microfluidic system for *in situ* Padlock probe rolling circle amplification (Padlock/RCA), a method of amplifying target DNA to visualize the intracellular DNA and improve the sensitivity and specificity of FISH procedures.[22] However, the multiple reaction steps involved make this technique costly and cumbersome compared to FISH. The automated microfluidic system could perform reaction solution exchange 13 times by means of a PC-controlled pump, which allows for reduction of labor-intensive manual operations (Fig. 3). Padlock/RCA was applicable to detect genetic abnormalities.[23] Padlock probes can distinguish single-base sequence differences,[22,24] which is not possible with the conventional FISH or in situ PCR. Moreover, *in situ* sequencing was also developed by combining padlock probes with next-generation sequencing methods.[25] Adding information about point mutations in cancer genome to morphological microscopic analysis allows visualization of intra-tumor heterogeneity, which may be important for tumor grading and classification.

4 Microfluidics for CTC Analysis

In general, FISH is performed on a tissue section isolated from a lesion site. Recently, testing methods for using CTCs in the blood as a new biomarker were developed to evaluate their potential in early cancer diagnoses.[5] The techniques necessary for testing CTCs in blood are isolation of rare CTCs from blood samples (usually in the rage of 1 – 100 cells/mL of blood) and single cell-based molecular diagnosis. Once the CTCs are isolated, FISH or a single-cell sequencing method[26,27] is applicable for carrying out single-cell molecular diagnosis. Thus, an effective method for the isolation of rare CTCs is a remaining key issue. The use of a microdevice is expected to be appropriate for highly efficient CTC isolation.

There are two microfluidic approaches for isolating rare CTCs

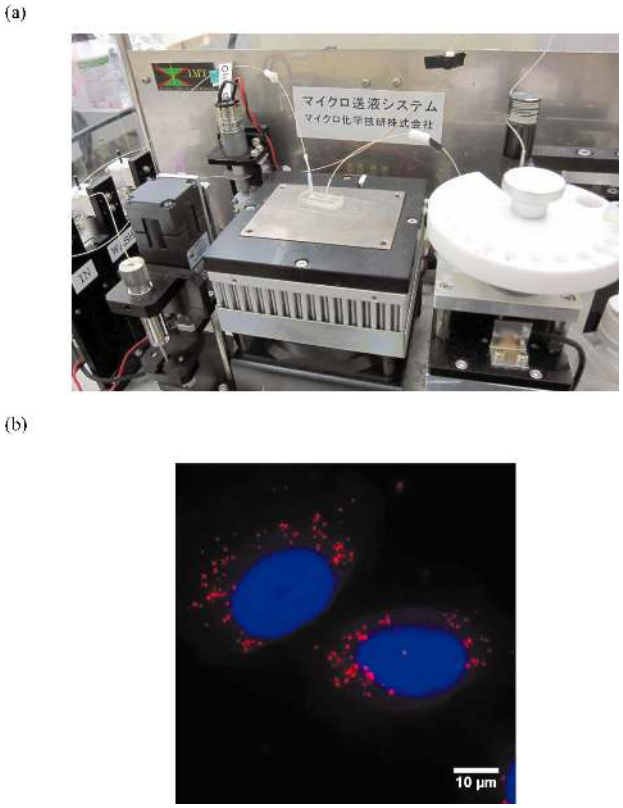

Fig. 3 An automated microfluidic system for integrated padlock/rolling circle amplification (RCA) reactions.
(a) Photograph of the system. (b) Detection of mitochondrial DNA fragment. Mitochondrial DNA (red) was amplified in HeLa cells using the microfluidic padlock/RCA reaction system. Cell nuclei are visible as blue staining.

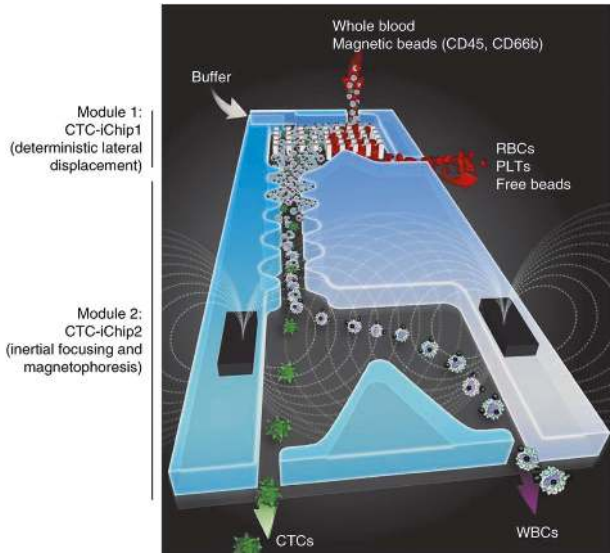

Fig. 4 Principle of circulating tumor cell (CTC) isolation from a patient's blood sample using the CTC-iChip composed of two separate microfluidic devices: deterministic lateral displacement (DLD) to remove red cells, plasma, and free magnetic beads from whole blood by size-based deflection using a specially designed array of posts performed in CTC-iChip1; and inertial focusing to line up the cells in preparation for precise magnetic separation and magnetophoresis for sensitive separation of bead-labeled white blood cells (WBCs) and unlabeled CTCs, performed in CTC-iChip2. Reprinted with permission from Ref. 31.

from blood samples: (1) size-based selection (large CTCs and small white blood cells [WBCs]), and (2) surface marker-based selection (usually EpCAM). Li *et al.*[28] demonstrated the effective separation of rare cancer cells (with average diameters of 16 or 20 μm, depending on the cancer cell lines used) from WBCs (diameter ~12 μm); the cancer cell recovery rate was >83% and the WBC removal rate was ~90%. The method is expected to work very well for cancer cells that show a significant size (or density/compressibility) difference between CTCs and WBCs. For cancer cells that have similar physical properties to WBCs, the method is considered to be less effective, and optimization of a specifically designed device would be required.

The other widely used CTC isolation technique is derived from antibody-based capture of CTCs, which express epithelial cell-surface markers that are absent from normal leukocytes. One of the pioneering studies of CTC isolation was based on the presence of the epithelial marker EpCAM and the absence of the CD45 immune surface receptors, which was realized using the CellSearch magnetic bead system (Veridex).[5] To enhance efficiency of detection, a magnetic beads system was integrated into the microchannel.[29–31] Karabacak *et al.*[31] developed an isolation method of rare CTCs from blood samples by using deterministic lateral displacement (DLD) and magnetic beads with a leukocyte-specific antibody. The device was composed of two separate microfluidic devices: Module 1, using DLD to remove red cells, plasma, and free magnetic beads from the whole blood by size-based deflection; and Module 2, involving inertial focusing and magnetophoresis for the separation of bead-labeled WBCs and unlabeled CTCs (Fig. 4). They achieved a 97% yield of rare cells with a sample-processing rate of 8 mL of whole blood per hour.

Furthermore, CTC isolation devices have also been developed in which the microchannel was modified with cell-capture antibodies. Nagrath *et al.* reported a CTC chip that had a silicon chamber with 78000 etched microposts that were coated with an anti-EpCAM antibody.[32] In this method, no pre-labeling or processing of the samples was required. The capture efficiency was further improved by adding micromixers and nanoscale structures on the substrates. A substrate with an antibody-coated silicon nanopillar structures was overlaid with staggered herringbone micromixers for fluid mixing, which was exploited for enhancing the cell capture efficiency.[33,34] Similarly, a method for the non-biased isolation of cancer cells (negative selection) in a peripheral blood sample was reported, which utilized microfluidic mixing PDMS devices that were functionalized with anti-CD45.[35] Introduction of micro- and nano-scale roughness using a single-step treatment with sulfuric acid significantly increased the binding yield of WBCs to the anti-CD45-conjugated surfaces. Up to 99.99% WBC removal was achieved with a tumor cell recovery yield of 50%.

After the CTC isolation process, the single-cell array format is convenient for analysis. Liu *et al.*[13] presented a method of preparing single-cell arrays for DNA FISH, which is based on chemical micropatterning on a flat surface to create an array of cell-adhesive islands (APTES-modified area) and a cell-repelling background (PEG-silane-modified area), followed by passive seeding of cells. This is a simple and inexpensive method, which allows for easy adaptation of conventional FISH protocols. Moreover, the surface chemistry and geometries of the array substrate were selected and designed specifically for

FISH. They used this method to create centimeter-sized single-cell arrays of nonadherent human cells, performed DNA FISH on the arrays, and analyzed the results with a computer program called FISH Finder specifically developed for FISH data analysis.

5 Conclusions

Here, I reviewed the feasibility of pathology analyses based on microfluidics, with particular focus on FISH and CTC analyses. An important goal in FISH protocol development is to reduce the assay time without compromising sensitivity and reproducibility. Microfluidics is a suitable technology for this purpose. Automation of the experiments realized by use of a microfluidic system with a pump has resulted in high reproducibility and reduction of the hybridization time with FISH probes. Moreover, consumption of DNA probes was reduced as compared to the use of glass slide protocols. However, in order for these methods to be practically and widely applied, development of cheaper devices and a high-throughput system that can simultaneously analyze many samples is required, which is a common issue in the micro-TAS (micro Total Analysis Systems) field.

Microfluidics is also a useful method for isolation of CTCs, a new biomarker, with highly efficient cell recovery (see Alix-Panabieres for a review on CTC analysis methods with and without microfluidics).[36] Diagnosis methods suitable for the personalized therapy of cancers have greatly improved in recent years. Since molecular-targeted drugs are preferentially used in cancer therapy, genetic testing methods at the single-cell level are required. Microfluidic cell analyses could greatly contribute to future medicine as rapid and reliable pathological diagnosis methods.

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