Lab on a Chip

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Title:

FISH in Chips: Turning microfluidic Fluorescent In Situ Hybridization into a quantitative and clinically reliable molecular diagnosis tool.

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Abstract:

Microfluidic systems bears promise to provide new powerful tools for the molecular characterization of cancer cells, in particular for the routine detection of multiple cancer biomarkers using a minute amount of sample. However, taking miniaturized cell-based assays into the clinics requires the implementation and validation of complex biological protocols on chip, as well as the development of disposable microdevices produced at low cost. Based on a recently developed microfluidic chip made of Cyclo Olefin Copolymer for cell immobilization with minimal dead volume and controlled shear stress, we developed a protocol performed entirely in liquid phase, allowing the immobilization and fixation of cells, and their quantitative characterization by fluorescence in situ hybridization. We demonstrated first on cell lines, and then on two clinical case studies, the potential of this method to perform quantitative copy number measurement and clinical scoring of the amplification of the *ERBB2* gene, a decisive biomarker for the prescription of HER2+ related targeted therapies. This validation was performed in a blind protocol on two clinical case studies, in reference with the gold standard and clinically used method based on glass slides. We obtained a comparable reproducibility, and a minor difference in apparent amplification, which can be corrected by internal calibration. The method thus reaches the standards of robustness needed for clinical use. The protocol can be fully automated, and its consumption of sample and DNA probes is reduced as compared to glass slides protocols by factors at least 10. Total duration of the assay is divided by two.

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Introduction

With the development of personalized medicine and targeted therapies in oncology, cancer patient management is now driven by the molecular alterations, mainly somatic mutations, detected in the tumor cells. A paradigmatic case is the *ERBB2* gene (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2), located in chromosome 17. This proto-oncogene, involved in the regulation of cell growth and proliferation, is amplified in about 15% of breast cancers. This amplification leads to the overexpression of the corresponding protein on cell membrane named HER2[1]. Breast cancers harbouring *ERBB2* amplification are usually of high tumor grade and were historically associated with a poor prognosis. In the early 2000's, trastuzumab, a monoclonal antibody directed against the extracellular part of HER2, demonstrated a very high anti-tumor efficacy. Other anti-HER2 therapies have been developed for this subgroup of breast cancers, whereas trastuzumab was also shown to be effective in other *ERBB2*-amplified tumors, such as in a subgroup of metastatic gastric cancers. HER2 status assessment is currently part of the daily routine management of breast and gastric cancers.

The assessment of HER2 status on tumor tissue is strictly codified by the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) guidelines[2]. Membranous HER2 protein over-expression can be assessed by immunohistostaining (IHC) or In-situ Hybridization (using fluorescent (FISH) or chromogenic (CISH) probes). IHC is semi-quantitative at best, and since HER2 is also expressed in non-amplified cells to a weaker extent, it yields ambiguous cases, with a suboptimal inter-reader reproducibility. Moreover, this technique is considered as not reliable for cytological samples. In situ hybridization is in contrast a quantitative detection method that allows determining the exact number of copies of the *ERBB2* gene per chromosome 17 on a cell-by-cell basis (ERRB2/SE17 ratio)[3]. Indeed, to distinguish an increase in the number of chromosomes (polysomy) from a real *ERBB2* amplification, targeting the centromeric region of chromosome 17 (SE17) used as an internal control is crucial.

Thus, to provide a valid assessment of the HER2 status of cytological samples, FISH is used as the "gold standard". However, the use of FISH in routine clinical practice has remained limited, in spite of its recognized superiority and inter-reader reproducibility over IHC. This is largely due to its high cost and technically demanding character, despite the use of robots for the numerous sequential incubations of glass slides required in conventional protocols.

Implementing FISH analysis on microfluidic platforms has gained a lot of interest, since it offers the possibility to create more compact and low cost automated platforms, while reducing the consumption of sample and reagents, in particular DNA probes. Being able to perform all the steps of the FISH protocol, from sample preparation to detection, in a closed chip can also help to reduce the risk of contamination or loss of precious samples. However, building an integrated and quantitative device for FISH analysis of real samples remains challenging: first, as for most cell-based assays, sample preparation on chip must be performed with caution, in order to immobilize cells on the surface with a high cell density, while avoiding cell overlapping. Shear stress should also be reduced during this step to limit the risk of cell disruption inside the confined channels. Second, the design and fabrication of the microfluidic platform should be optimized to allow the delivery of precise volumes of complex mixtures and reagents with different viscosities into the reaction chamber in a serial manner, without inducing cross contamination; precise temperature control is also an important parameter during the different steps of the FISH protocol. Finally, the chip should be compatible with high magnification fluorescence imaging for efficient FISH signal detection and quantification.

Taking in consideration all these technical requirements, up to now only few examples of proof-of-concept miniaturized FISH platforms have been proposed in the literature[4–8]. Their primary focus has mostly been technological, showing different strategies to reduce the cost of the test by reducing the volume of all FISH reagents, in particular DNA probes (the most expensive reagent) required per sample (by 10 to 30-fold) as well as the labor time (by 10-fold). The pioneer work of Sieben et al.[4] was based on the integration of elastomeric valves, pumps and thin-film platinum heaters inside narrow

microchannels (chemically etched in glass substrates) with low dead volume, in order to build a fully automated FISH platform. In this work, however, the fabrication process involved complex technologies and required the use of a clean room environment increasing the cost of the test (100 $ per chip). Using an integrated heating system can be an advantage for local and precise temperature control but it also requires important technical skills for a proper calibration prior to each use. A similar automated platform has been proposed more recently[7], employing a suction-type pump to deliver samples and reagents to a dedicated reaction chamber, while controlling their temperature using an external thermoelectric cooling module. These improvements decreased to some extent the sophistication level of the device and thus the fabrication cost (based on soft-lithography techniques), but the multilayer process required during the assembly of the micropump remains time-consuming. Another strategy aiming at improving the robustness of the cell immobilization step on the chip surface, has been developed using a simpler device made with PDMS microchannels reversibly bonded to nanoengineered glass slides[5]. This architecture was very efficient in enhancing the adhesion and confinement of cell suspensions, thus offering the possibility to enrich samples with low cellular content. However, the procedure could not be fully automatized since it required manual handling and removal of the PDMS lid to complete the FISH protocol following the conventional procedures for glass slides, thus reducing automation potential, and increasing the risk of sample contamination. Moreover, this approach requires expensive and complex fabrication procedures involving glass slides with TiO_2 assembled nanoclusters. Finally, a recent study has proposed to implement micropatterning based methods to create a functionalized array of “cell-adhesive” islands on glass slides and enable the precise positioning of single cells to expedite the image acquisition step[8]. Although, this work demonstrates the possibility to perform large-scale high-throughput FISH analysis, no modification of the standard FISH protocol was performed and the cell patterning process remains laborious.

These examples demonstrate the benefit of microfluidic devices for cellular assays, but they mostly ignore another important aspect in the transition of microfluidic protocols to the clinical world, i.e.

chip cost. Indeed, it is no use reducing reagents consumption, if this gain is lost in the additional cost of the chip production, operation or storage, as compared to a glass slide (the standard support for FISH analysis). PDMS, for instance is very popular for fast prototyping, but it presents many inherent drawbacks[9] (e.g. high water and gas permeability, swellability by most organic solvents, notably alcohols). These features make its use inconvenient for FISH assays, which require the use of solvents and thermal treatments. The production of PDMS chips is also rather expensive. In contrast, thermoplastic materials such as Cyclic Olefin Polymers (COP) and Cyclic Olefin Copolymers (COC) present many assets for future routine clinical use[10–13]. They are available at very low cost (typically in the order of a few $ per kg for bulk raw material), in different grades resistant to harsh solvents and temperatures up to 180°C. They also present excellent optical quality in the visible and UV ranges, allowing for high quality fluorescence imaging and they exist in grades approved for clinical use. Last but not least, they are amenable to high resolution and high throughput microforming by hot embossing or injection molding for routine mass-production of monolithic and fully disposable devices. The small scale production of COC chips with custom designs still require an initial investment[14,15], but routine production can drop costs dramatically, typically to a few $ per chip or less.

Thus, in our aim to develop a device transposable to routine clinical use, we selected COC as the fabrication material, and a design previously conceived by our group to solve some of the problems mentioned above[16]. This device combines a simple design, small footprint and allows the implementation of standard biological protocols in a chip format with low volume consumption. The chip design was optimized from a hydrodynamic point of view to provide a flexible and efficient platform for the immobilization and analysis of cells. This new design also allowed us to obtain a high density monolayer of intact and non-overlapping cells, which is crucial for cell based assay. This feature was achieved thanks to the laminarity of the flow and to the absence of stagnation areas in the chamber, the latter arising from its 3 dimensional “slanted” walls. We also demonstrated the

possibility to perform FISH imaging. However, this earlier version did not have the automation or the quantification power needed for clinical scoring applications.

Thus, so far none of the previously described microfluidic FISH platforms and protocols, including ours, has been pushed to a level of robustness and quantitativeness suitable for clinical applications. They involved proof-of-concept experiments with a direct transposition of the standard FISH protocol, providing "yes or no" results with no quantitative analysis of gene amplification and scoring, as needed e.g. for the assessment of *ERBB2* amplification.

In the present work, we try to bridge this gap, and build upon our earlier successful proof of concept[16], to develop a complete, simple on-chip FISH protocol suitable for quantitative molecular typing in a clinical setting, while keeping low production and operation costs. As an application, we selected the quantitative assessment of the number of copies of the *ERBB2* gene, and of a reference in the centromeric region measuring polysomy of chromosome 17. As recalled in the literature, this typing is currently of paramount importance in treatment orientation for breast cancer patients: originally, the *ERBB2* gene amplification has been associated with aggressive tumors and a negative, more pessimistic prognostic for patients treated with conventional methods. Targeted therapies for these types of tumors have been developed, however, and have considerably improved the outcome and prognostic for these patients. Thus, we believe that this application constitutes both a route to a rapid and extremely useful new application of microfluidic technologies in real life, and a challenge to promote the maturation of lab on chip technologies, and demonstrate their potential in applications as demanding as clinical diagnosis.

We first quantified the HER2 status of two different cancer cell lines through high resolution microscopy, and compared the results with those of a conventional protocol on glass slides considered as the current gold standard. We also evaluated the scoring provided by our method regarding two samples from breast cancer patients.

Experimental Section

1. Materials

Cyclic olefin copolymer (COC) films (Topas® 8007, T_g = 80°C, 145 µm thick) and plates (Topas® 8007, T_g = 80°C, 5 mm thick) were purchased from Topas Advanced Polymers, Extrusion Lab (Germany). 2-Hydroxyethyl cellulose –HEC – (average Mv ~90,000), Poly-L-lysine –PLL – solution (mol wt 150,000-300,000, 0.01%, sterile-filtered) and pepsin lyophilized powder (from porcine gastric mucosa; 3,200-4,500 units/mg protein) were purchased from Sigma-Aldrich (USA). The ready-to-use FISH probes (ON ERBB2, HER2/*neu* (17q12) / SE17), the FISH Hybridization Buffer (FHB), DAPI (4',6-diamidino-2-phenylindole) counterstain (1µg/mL) and counterstain diluents were purchased from Kreatech Diagnostics (The Netherlands). SuperFrost® Plus glass slides were purchased from Menzel-Gläser (Germany). Cell culture reagents were purchased from Gibco, Life Technologies (USA).

2. Microfluidic device design and fabrication

The details and hydrodynamic characterization of the chip are described elsewhere[16]. In brief, the chip design (Figure 1 a) consists in a dual structure comprising narrow channels (height=30 µm, width=60 µm) and large 3D chambers (height=0.38 mm, bottom diameter=1 mm). The device was prepared in COC plates using a hot embossing procedure as described [16,17]. A solvent assisted plasticizing process [18] was used to bond a thin COC film to the embossed microfluidic plate (25mm × 35mm and 5 mm thick), thus providing a monolithic, fully disposable chip. The COC film has a thickness (145 µm) comparable with that of glass coverslips, in order to allow high resolution imaging with objectives up to 100X oil immersion.

In our previous work, fluid handling was performed manually[16]. Here, to increase reproducibility and prepare for a clinical environment, this chip was associated with a fully programmable pressure-based fluid handling platform. Tygon (Cole-Parmer) and Polytetrafluoroethylene (PTFE) tubes (inner diameter: 0.028 mm) were used for the fluidic connections. The COC chip was coupled with a flow

control system (MFCS, Fluigent) for the injection of all reagents. A double layer coating was used for the surface treatment of the COC microchannels to render the surface hydrophilic and avoid non-specific adsorption of proteins[19] , and at the same time to enable the attachment of the cells to the bottom surface of the 3D chambers[20] (Figure 1 b). The procedure was the following: first, 200 µL of HEC solution (2 % wt in Phosphate-Buffered Saline -PBS- 1X) were injected in the channels and let to incubate (1h at room temperature -RT-). After a rinsing step with PBS, the microchannels were filled with 100 µL of PLL, let to incubate (3 hours at 37°C) and washed with PBS prior to the injection of cells.

The thermal control of the chip was provided by a high precision hot plate (Stuart Equipment, UK) coupled with a temperature probe (Implementable Thermocouple probe, Thermo Fisher Scientific) that was inserted in the chip.

3. Cell culture:

The human epithelial cell lines SKBR-3 (*ERBB2* amplified breast adenocarcinoma) and G-401 (*ERBB2* unamplified rhabdoid tumor) were obtained from the American Type Culture collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL aqueous penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine, at 37°C in a humidified atmosphere with 5% of carbon dioxide.

4. Sample preparation:

For cell lines, the confluent culture cells were detached by a trypsin treatment (0.05% Trypsin-EDTA 1X), centrifuged and re-suspended in 1mL culture medium to obtain an approximate concentration of 10^6 cells/mL. For the standard FISH protocol, five glass slides were placed at the bottom of a Petri dish (10 cm in diameter) filled with 25 mL of culture medium. 180 µL of cell suspension were added on top of each glass slide by manual pipetting, covering the entire surface of the glass slide. The cells were

then allowed to adhere properly and spread on the glass slides, overnight (37°C with 5% CO_2). The culture medium was finally removed, and the slides were washed with PBS 1X buffer two times.

The remaining cell suspension (100 µL) was kept at room temperature for at most 5 minutes, and the desired volume was injected in the COC chip for the miniaturized FISH experiments. The procedure for cell immobilization on chip has been previously described[16]. It is based on the use of a PLL surface treatment to enable cell adhesion on the COC surface. This was combined with a novel 3D chip design which creates strong flow velocity contrasts between the deep chambers and the low profile connection channels. This allows confining cells over a restricted area (with low flow velocity) whereas no cells remain in the connecting channels (Figure 1 a). The design also allows a minimal dead volume on the chip thanks to the small size of the connecting channels and of the chambers (total volume of 2 µL), avoidance of stagnation points in the flow, and optimal fluid replacement with a minimal rinsing volume. The workflow follows three different steps: first, cell injection, then an incubation step (5 minutes with no flow rate) during which cells are allowed to adhere to the surface; finally a washing step with PBS 1X buffer to remove non adhered cells.

Pleural effusions: two different samples were obtained from pleural effusions from two *ERBB2*-amplified metastatic breast cancer patients. These patients presented with dyspnea due to metastatic pleural effusion and had pleural punctures as part of their usual clinical management. Following patients consent, pleural effusions were transferred to the laboratory instead of being discarded. Samples were analyzed in parallel in a blind protocol, by a combination of cytology (May-Grünwald-Giemsa stain) and FISH performed on glass slides on the one hand (at the department of Pathology, Institut Curie), and by FISH using the COC chip on the other hand, without prior knowledge of the nature of the cell content in the samples. Prior to the FISH on-chip analysis, red blood cells, polynuclear cells and plasma were removed using a Ficoll density gradient, and the mono-nucleated cells were obtained in 100 mL of sample. This enriched sample was re-suspended in PBS 1X to reach a total volume of 500 µL and 15 µL of the cell suspension were injected in the chip for FISH analysis.

5. Fluorescence in situ hybridization (FISH) protocol

The efficiency and sensitivity of the FISH protocol were first validated using cells immobilized on glass slides. This conventional protocol was used as a reference in terms of signal quality, copy number enumeration and preservation of cell morphology during the implementation of the FISH protocol on chip. Glass slide experiments were performed in parallel for each FISH experiment on chip as a control.

Slides were first incubated in SSC 2X buffer (diluted from SSC 20X, pH~7) at 37°C for 2 minutes. The samples were pre-treated with pepsin (0.05 µg/mL in 0.01 M HCl) for 7 minutes at 37°C, washed in one change of PBS 1X and one change of SSC 2X at room temperature. The slides were then fixed with Carnoy's Fixative (Acetic acid/ethanol 1:3 (v/v)) at 5°C during 20 minutes and then rinsed with SSC 2X two times (10 minutes at 37°C and 2 minutes at room temperature). The cells were then dehydrated by ethanol (respectively with 70%, 90% and 100%, 2 minutes for each solution) and dried at room temperature. The hybridization mix (10 µL of labelled DNA probes) was applied on the slides and covered with a glass coverslip. DNA probes were co-denatured at 75°C for 15 min and then incubated at 37°C overnight in a humid atmosphere. Unbounded/non-specifically bounded probes were removed by standard procedures : first in washing solution I (0,4X SSC +0.3% NP40, pH~7) for 3 minutes at 72°C and then in washing solution II (2X SSC +0.1% NP40, pH~7) for 3 minutes at room temperature. Finally, following the dehydration step, 10 µL of DAPI (diluted at a concentration of 0.2 µg/mL in counterstain diluent) was applied on the cells and covered with a glass coverslip.

For the implementation of the FISH protocol on chip, we miniaturized the standard procedure (Table 1) and performed several modifications of the standard protocol to gain a better compatibility with our system (See Results and discussion section for rationale). New alternatives were developed (Table 1, steps 7 and 12) to implement the FISH protocol in a flow through format, taking inspiration from[21]. The short washing steps involved in glass slides protocols were also replaced by a continuous flow washing step on chip to increase efficiency. The concentration of pepsin solution for the enzymatic digestion step (Table 1, step 3) was increased from 0.05 µg/mL to 1 µg/mL. Finally, the 10 µL of FISH

probes were replaced by 2.5 µL of probe diluted in 2.5 µL of FHB, contributing to a strong reduction of reagents costs. The duration and temperature of the different incubation steps were kept constant. For the analysis of cells from the pleural effusions on chip, a supplementary step of RNAse treatment (0.1mg/mL, at 37°C for 1 hour) was added after the fixation step. It was followed by two rinsing steps, first at room temperature with 100 µL SSC 2X (200mbars) and then at 37°C for 10 minutes in SSC 2X.

6. Image acquisition and treatment

Imaging of the labelled cells on glass slides and on chip was performed using an epi-fluorescence microscope (Nikon Eclipse 80i) equipped with a piezo focus lens positioner (P-721 PIFOC®, Germany), a high speed CCD camera (CoolSNAP HQ2, Photometrics, Roper scientific - Princeton instruments), a mercury lamp (HGFIL Lampe 130 W) and adequate filter sets (DAPI, GFP, RHOD). The images were taken using an oil immersion 100X objective (Nikon, CFI Plan Apo VC, NA 1.4, WD 0.13). Fluorescence images for each set of filters were recorded using the MetaMorph® Imaging Software (Molecular Devices). The step of the piezo scanner was set at 0.2 µm in order to acquire high resolution 3D images (z stacks, 5-20 µm scan total length) (Figure 1 b). Before image analysis, the "Meinel" algorithm for 3D deconvolution of fluorescence signals was applied to the recorded z stacks[22].

7. FISH probes and HER2 scoring criteria

According to 2013 the ASCO/CAP guidelines[2], gene amplification using dual-probe assays is defined as a *ERBB2*/SE17 ratio ≥ 2.0 with an average *ERBB2* copy number ≥ 4.0 signals. In the presence of chromosomal abnormalities, like aneusomy of chromosome 17 (polysomy and monosomy), samples with a *ERBB2*/SE17 ratio < 2.0 will be considered as amplified only if they present an average *ERBB2* copy number ≥ 6.0 signals. If the average *ERBB2* copy number is < 4.0 signals the sample will be negative. Other rare or equivocal cases are explained in detail on the ASCO/CAP guidelines[2]. Thus, both *ERBB2* and SE17 copy numbers must be quantified in each cell. The *ERBB2* specific probe is labelled with a red dye (PlatinumBright 550) and the control DNA probe for the centromere of

chromosome 17 (SE17) is labelled with a green dye (PlatinumBright 495). For the FISH experiments on chip, the FISH probe was diluted at the desired concentration using FHB.

FISH analysis was performed by fluorescence microscopy as described above. Only single, non-overlapping and intact nuclei were scored. For each nucleus, red and green signals were counted separately using the ImageJ software (http://rsb.info.nih.gov/ij). The *ERBB2* amplification score was calculated as the ratio of the total *ERBB2* signals to the total SE17 signals counted on a single cell basis, over at least 20 different cells for each experiment.

Results and Discussion

1. Technological transfer of FISH protocol from glass slide to COC chip

Implementing a cellular assay in a microfluidic format often requires redesigning the protocol of the bioassay. Among cellular assays, FISH is considered as a complex and demanding process. It consists of at least seven steps involving the use of organic solvents, dehydration and drying, heating combined with enzymatic digestion as well as DNA hybridization. A direct transposition of the FISH protocol performed on glass slides did not yield reproducible and accurate enough results, and a major reconsideration of the protocol was necessary.

The development and optimization of the on-chip protocol were performed with cell lines and using a FISH probe targeting the centromeric region of chromosome 17. The diploid G-401 cell line was used as a control. According to the supplier recommendations, an efficient FISH protocol should result in round, smoothly defined and correctly stained nuclei giving bright, compact and discrete fluorescence signals. The absence of non-specific hybridization signals or background fluorescence is also an important criterion for successful signal enumeration. For FISH experiments with the G-401 cell lines, two bright spots should be present inside each nucleus. Split signals in very close proximity, typical of cells having passed through the S phase of the cell cycle, were counted as one signal.

1.1. Flow-through "all wet" FISH protocol on chip

Several parameters were modified to optimize the standard FISH protocol with regards to the specific requirements of miniaturization. A major concern in miniaturized assays is the increased importance of surface properties, a consequence of the higher surface to volume ratio. As an illustration, a 20-fold increase in the enzyme concentration was necessary to circumvent the non-specific adsorption of these molecules to the COC surface, and efficiently digest the proteins of the immobilized cells. The duration of each step in the protocol was also modified as compared to the glass slide protocol, to take into account the different kinetics induced by miniaturization and by the continuous flow-

through nature of the incubation. Temperature, flow rate and buffer composition were also optimized (data not shown). Here we only present in more detail a protocol modification that was crucial for the implementation of a flow-through protocol in closed COC microchannels.

In a standard FISH protocol on glass slides, cells are dehydrated by a series of ethanol solutions (e.g. 70%, 90% and 100%) and dried (Table 1). Dehydration by ethanol is expected to promote DNA condensation and promote its firm adhesion to the surface. It is also expected to help to eliminate any traces of buffer or reagent (e.g. fixative solution) that may inactivate the probes. After that, however, ethanol must be thoroughly removed, since it may hamper hybridization and proper diffusion of the FISH probes through the nucleus. From a practical point of view, on glass slides it is also simpler to seal the probe under a cover slip with rubber cement when the slide is dry.

Indeed, most of earlier on-chip protocols retained a drying step. This has been achieved by opening the chip during part of the protocol[5,6,8] and returning to an "open air" protocol close to that used on glass slides. We wanted to avoid this approach, which strongly reduces the potential of microfluidics for full automation. Sieben et al.[4] performed the whole protocol in a closed channel, but flushed air in the chip. In our hands and in our COC chip, this approach did not yield reproducible enough results, and we identified several possible reasons for that: at these small scales, wetting phenomena are critical, and it is difficult to prevent residual fluids to remain in the chip, especially in corners, when air is pushed through. This can yield poor rinsing, crystal deposition, and even clogging problems. Also, controlling the dehydration and drying steps inside COC chips remains challenging and prone to artefacts. As most thermoplastics, COC is not permeable to gas, and the drying process through ethanol evaporation in closed channels is very slow (up to a few hours at room temperature). The consequence of a partial drying can be dramatic, since the presence of ethanol in the channels results in the aggregation and degradation of the DNA probes, decreasing the FISH efficiency (Figure 2 a). PDMS could be better than COC in this respect, since it adsorbs ethanol quite extensively, but then the ethanol may be slowly released in the chip during the remainder of the protocol, in a poorly

controlled manner, and affect the efficiency and reproducibility of hybridization. Besides, as discussed in the introduction, we wanted to avoid PDMS, which is not a good candidate for routine clinical use.

To overcome the above problems, we proposed a new FISH protocol where ethanol dehydration steps are replaced by a washing step with SSC 2X buffer at room temperature. We evaluated the performance of this new protocol performing microfluidic FISH on G401 cell lines and comparing it to the standard FISH protocol on glass slides. The results showed that we obtained a robust and reproducible FISH protocol in the COC chip (counted signals per cell ± S.D.: 1.98 ± 0.15). This result is comparable with the control on glass slides (Figure 2 b) (counted signals per cell ± S.D.: 2 ± 0). We did not observe any cell with more than two SE17 signals, showing that the hybridization was specific and that the post-hybridization washing step was efficient to remove non-specifically bounded probes. This is an important result since we were able to successfully adapt the FISH protocol to our system by modifying a critical step, without decreasing the efficiency of the hybridization or inducing any change in the morphology of nuclei.

1.2. Reducing the amount of FISH probe and the cost per test

Once the on-chip protocol has been established, we took advantage of miniaturization to decrease the final cost of the FISH analysis. The use of microfluidics has provided a drastic reduction of the volume of all reagents (SSC 2X buffer, protease and fixatives solutions, washing solutions I and II, counterstaining and mounting medium) from the milliliter to the microliter scale (Table 1). This not only reduces further the cost of the test but it also facilitates the handling of fluids and eliminates some fastidious steps of the FISH protocol.

We also investigated the possibility to decrease the required amount of probe. Indeed, fluorescently labelled DNA probes are the most expensive reagent of the FISH assay (10 µL of probe at 1x are needed for one experiment on glass slide), as compared to the other reagents. With the design proposed in this work, the volume of probe needed to perform one test was reduced to 2 µL (5 fold reduction). In order to reach submicroliter amounts of probes, previous works have proposed to

decrease the inner volume of the chip[4,5]. However, this requires to drastically reducing the dimensions of the channels, thus generating higher shear stress that may severely damage cells. Therefore, we propose here an alternative strategy, taking advantage of the faster and better controlled kinetics achieved in flow-through miniaturized chips, to further decrease the amount of probe required, while keeping shear stress to a harmless level. We investigated the efficiency of serial dilutions of the probe as compared to the concentration usually used on glass slides ([C]=1x). Three different concentrations of probe were investigated on chip ([C]=1x, [C]=0.5x and [C]=0.25x). To characterize the efficiency of probe hybridization for each concentration in the FISH protocol on chip, we evaluated the signal-to-noise ratio (SNR) of the SE17 signals and compared it with the SNR of signals from cells analyzed on glass slides. SNR measurements comparing the fluorescence intensity from the FISH signals and the background fluorescence in the area of the nucleus outside the FISH signals can be used as an objective method to compare the quality of fluorescence signals in FISH analysis, and to measure the intensity variability of the FISH signals from cell-to-cell and from two different sets of experimental conditions[23].

Regardless of the probe concentration, all the scored cells contained two clearly distinguishable and bright SE17 signals (Figure 3 b, c and d), showing no loss of signals as compared to the standard concentration of probe on glass slide (Figure 3 e). The signal-to-noise ratio was also equivalent for all the different probe concentrations on chip (Figure 3 a), as compared to the control glass slides ([C]=1x), showing that the hybridization efficiency was well preserved. Interestingly, similar reduction of probe concentration in the conventional glass slide protocol (which is not recommended by kit providers) yielded an increase in the number of the poorly labelled and non-analysable cells (data not shown). The possibility to use, without such damage lower probe concentration in our chip protocol *a posteriori* justifies our assumption, that the laminar flow pattern in our microfluidic system yields a better efficiency and reproducibility of probe transport and renewal, and thus better hybridization at lower probe concentration. This high efficiency could also be a consequence of the absence of a drying step in our protocol: drying induces a collapse of all macromolecular structures, including

DNA, into a compact highly entangled state, promoting the formation of irreversible bonds or micro-aggregates. Thus, upon re-swelling with the hybridization solution, genomic DNA probably does not recover the same availability and permeability as before drying. It is then plausible that both efficiency and kinetics of hybridization could be lower than with a "fully wet" protocol.

Considering that the volume of the chip is 2 µL, the three different concentrations that have been tested correspond to a reduction in the amount of probe needed for one sample by 5, 10 and 20-fold, respectively, with no significant modification of the signal-to-noise ratio as compared to the "gold standard". As compared to previous works dealing with the miniaturization of FISH protocols on chip[4,5] this is an important step towards a significant reduction in the cost of the assay while preserving its quality.

2. HER2 scoring by FISH: COC chips vs. glass slides

In order to take miniaturized cell bioassays into the clinics and take over conventional analysis tools, it is important to go beyond proof-of-concept FISH experiments on chip[4–8] that show only qualitative gene identification and check the potential of our microfluidic platform and optimized protocol for quantitative scoring.

2.1. Validating the FISH assay using cell lines

Diagnosis requires to clearly distinguishing a polysomy of chromosome 17, on which trastuzumab based treatments bring no advantage, from a real *ERBB2* gene amplification, defined as the presence of multiple copies of the gene per chromosome 17. In order to evaluate the potential of our method in this respect, we first used two cell lines representing different situations. The G-401 diploid cell line is used as an "unamplified" control, without polysomy of chromosome 17. These cells must present two red and two green dots (*ERBB2*/SE17 ratio equal to 1). The SKBR-3 cell line was used as a model for gene amplification (*ERBB2*/SE17 ratio greater than 2). SKBR-3 is a hypertriploid human cell line with a modal chromosome number of 84, occurring in 34% of cells (American Type Culture Collection

website). Consistent with previous reports[24], we measured for these cells an average of 7 copies of chromosome 17 and more than 30 copies of the *ERBB2* gene per cell.

Representative fluorescence images of the FISH analysis for HER2 scoring are depicted in Figure 4. They were performed for both cell lines immobilized on glass slides and on the COC chip. The flow-through FISH protocol provided bright and homogeneous SE17 and *ERBB2* spots on chip for both cell lines. The signal to noise ratio was comparable to the results on glass slides (Figure 4). FISH results on chip showed a low fluorescence background and no non-specific signals outside the nuclei, which are important requirements for reliable signal enumeration and HER2 scoring. The high image quality also shows the excellent optical properties of the COC material (no optical distortions and no autofluorescence) and the efficiency of the double-layer surface treatment. One also observes a good preservation of the nuclei morphology inside the COC chip, as compared to the glass slides, showing that the shear stress in the 3D chambers was maintained at harmless levels, and no cells were damaged or deformed during the experiment.

This new protocol involves a significant gain in the overall protocol duration: in the COC chip, cells can be immobilized and attached to the bottom surface of the 3D chambers in less than 15 minutes[16], without the need of thermal treatments or harsh fixatives that can over cross-link cell proteins and decrease FISH efficiency[8]. The conventional protocols on glass slides require an incubation of cells overnight to ensure cell attachment. The counterpart for this gain was a slight increase in the thickness of cells. The preservation of the three-dimensional shape of nuclei on chip, as compared to glass slides where the cells are present in a more “flat” configuration, has also been observed in other microfluidic platforms using other cell immobilization protocols[23]. This can be very useful during morphological cell analyses, but for the FISH application contemplated here, it requires to optimize the fluorescence imaging process and the image post-processing (Figure 1 b), in order to circumvent the possibility of FISH signals overlapping on the z axis. This is particularly critical for epithelial cancer cells, which can present important shape variations, and be much larger (diameter of around 40–42 µm) than e.g. normal or blood cells (diameter of 8–11 µm)[25]., and for cells with high

gene amplification levels such as SKBR-3 cells, but also cells from HER2 positive patients. These requirements have not been mentioned during the development of previous FISH platforms, using different DNA probes and targets, where only 2 to 4 signals needed to be analysed per cell [4,5], but they are crucial for efficient HER2 scoring. In our analysis, we thus used 3D deconvolution microscopy and analysed a stack of focal planes along the z axis. As compared to glass slides, the additional scanning time is compensated by the compactness and good distribution of the cells in the chip, which reduces the area to be scanned.

On a quantitative ground, the results for HER2 scoring on chip are presented in Figure 5, showing the average scores obtained using the G-401 and SKBR-3 cell lines. The error bars show the standard deviation from the scores obtained from n cells. We obtained a reproducibility of the FISH protocol on chip comparable with that of glass slides for both cell lines. For the G-401 cell line, the average *ERBB2*/SE17 ratio on chip corresponds to the expected value (*ERBB2*/SE17= 0.99 ± 0.07) and stands well below the *ERBB2* amplification threshold, in good agreement with the "unamplified" status of this cell line. For the SKBR-3 cell line, the average *ERBB2*/SE17 ratio for experiments on chip (*ERBB2*/SE17= 3.7 ± 1.3) was somewhat lower than the results obtained using conventional glass slides (*ERBB2*/SE17= 4.62 ± 1.1). However scores for both methods were well above the *ERBB2* amplification threshold given by the ASCO/CAP guidelines recommendation (*ERBB2*/SE17 ratio ≥ 2.0 with an average *ERBB2* copy number ≥ 4.0 signals) in good agreement with the "amplified" status of this cell line (Figure 5). The important cell-cell variability for the SKBR-3 cells, could be explained by the important genetic dispersion of this cell line[24,26].

Thus, as a summary, the reproducibility and reliability of scoring in the new on-chip FISH protocol is comparable with that of the conventional one on glass slides, but the measured amplification is in average slightly lower in the new protocol. As mentioned above, our SSC "all aqueous" treatment involves a faster and more "gentle" treatment of the cell's DNA, since there is no dehydration, and the incubation is much shorter. It was indeed noticed that cells inside the COC chip did present a more "round" configuration, as compared to cells adhered to the glass slide in the conventional

protocol. This may induce some differences in the availability of the target sequences, and thus a slight difference in measured amplification.

Also, although FISH on glass slides is currently considered the "gold standard", it may itself be prone to some bias. For instance, it may artificially over-evaluate the number of copies, as a consequence of the more "harsh" effect of the protocol (involving condensation, drying and re-swelling of nuclear DNA), which could split some positive chromosomal regions, and this artefact may depend on the target gene length and location. In any case, we insist, on the fact that this difference is consistent and reproducible, and the dispersion on data for the chip results is comparable with that obtained on glass slides and reminiscent of the previously reported standard deviation of this cell line[24]. Thus, it can be corrected by a suitable cross calibration, and will not affect the quality and significance of scoring.

We acknowledge that an equivalent error bar size, combined with a slightly lower apparent amplification, may increase the number of ambiguous cases. However, first the discrepancy is small, so the impact will be low. Second, the current amplification threshold of 2 is itself an empirical value, with a clinical rather than biological basis, and established with the conventional FISH method that is itself not absolute. Thus, we are confident that a well-conducted clinical validation of our protocol will be able to provide a correction to the threshold, which will restore a number of ambiguous cases not higher than that obtained with the current protocols. Finally, one may recall that the ASCO/CAP guidelines have already taken into account the possibility of ambiguous scoring and provided independent criteria to help clinicians in such situations[2].

2.2. Validating the FISH assay on chip using patient samples

After the above quantitative validation of the method with cell lines presenting (in average) a stable and known amplification score, we also demonstrated the possibility to perform our FISH on-chip protocol on real samples with different cellular contents in a complex matrix. As a proof of concept, we tested pleural effusions from two breast cancer patients. This liquid sample typically contains

mesothelial cells from the pleura mixed with tumor cells, platelets, red and white blood cells, proteins, and lipids. In some cases it can also show signs of infection, e.g. bacteria. As pleural effusion can be considered as a material representative of metastasis and given its complexity, it appears to be an interesting sample to evaluate the clinical performances of our microfluidic platform. It is important to notice that the primary tumor from both patients had been previously analysed by immunohistochemistry, and classified as HER2-positive.

We were able to immobilize several subpopulations of cells from the pleural effusion with different morphologies and sizes of nucleus (from 6 to 10 µm in diameter in the first sample and up to 20 µm in second sample) showing that the COC device is compatible with the use of real and complex clinical samples (Figure S1 in Supplementary Information and Figure 6 a). For the first patient sample (sample A), the analysis by FISH in our device did not show any *ERBB2* amplification, indicating that the immobilized cells were merely leukocytes or mesothelial cells (Figure S1). The cytological evaluation of the fluid performed in parallel did not show any evidence of malignant cells or infectious material. Therefore, no further FISH analysis was performed on glass slide by the pathologist.

For the second sample (sample B), we observed different populations of cells with clear and distinct *ERBB2* and SE17 signals, and no signs of cell deformation: some small cells (probably lymphocytes) presented no polysomy of the chromosome 17 and no *ERBB2* amplification (*ERBB2*/SE17 = 1); some big cells with *ERBB2* amplification but without polysomy, and some bigger cells with polysomy and *ERBB2* amplification (Figure 6 a). Cells in this second category (with or without polysomy) showed clear SE17 signals with clusters of *ERBB2* signals, which are considered to be a typical signature of high gene amplification [27,28]. Despite the good quality of the fluorescence images, the clusters for the *ERBB2* signals were too dense to allow for an accurate quantification of the number of copies, in order to provide an *ERBB2* amplification score. Concerning the polysomy of chromosome 17, most amplified cells only presented two copies, but some of the bigger cells presented 3 to 4 copies of the chromosome. Both cell types can be considered as tumoral cells and although the amount of cancer

cells with *ERBB2* amplification was low as compared to blood and mesothelial cells in the sample, the presence of malignant cells with HER2-positive status in the pleural fluid should provide clinicians useful prognosis information[29]. Interestingly too, the capacity to distinguish cells with a simple polysomy from cells with amplification in the same sample gives access to some information about cellular subpopulation heterogeneity, a question currently considered as extremely important for better understanding therapeutic escape and improve treatment. Such distinction is not possible with immunophenotyping.

Sample B was also evaluated in a blind process by experienced cytopathologists, using bright field microscopy after May-Grünwald-Giemsa staining, showing the presence of gigantic cells with tumoral features (data not shown). This first evaluation was followed by FISH analysis on glass slides, which detected *ERBB2* amplification in the tumor cells. The quality of the FISH signals obtained by pathologists was similar to our results on chip and the different cell types immobilized on glass slides presented the same features as those described above, in particular the presence of *ERBB2* clusters in HER2-positive cells (Figure 6 b). Thus, the immobilization of cells from a complex sample such as pleural effusion followed by the HER2 typing with FISH in our COC chip yielded an excellent agreement with the cytological and molecular analyses performed in the clinics for both samples. It shows that our approach could be as efficient as the “gold standard”, thus offering a promising potential for *in situ* analysis of cancer cells. This is the first time that a microfluidic platform for FISH analysis has been submitted to a blind protocol in the clinics to test its level of maturity. We are now working in further developments of this system to be able to perform a large scale study.

Conclusion

We have developed a microfluidic platform for Fluorescence In Situ Hybridization, able to provide quantitative scoring suitable for diagnosis. FISH is the gold standard to assess the presence of gene amplification (*e.g.* ERBB2), deletion (*e.g.* PTEN) and translocation (*e.g.* EML4-ALK fusion)[30] in tumor samples, and is used for diagnosis, prognosis and treatment orientation. Based on a new 3D chip design optimized to allow uniform cell adhesion and a strong reduction of volume as compared to the conventional methods on glass slides, we developed a new sample treatment and FISH protocol avoiding the dehydration and drying steps needed on slides. This eliminates several difficulties associated with drying in closed microchannels, and allows for the full automation of the protocol under fluidic control. We believe that this change was a major asset to improve the level of quantitation, reproducibility and accuracy of our approach as compared to previous microfluidic FISH protocols, and make this approach suitable for clinical diagnosis on real samples and in a routine setting.

This was demonstrated by applying this new method and chip system to the scoring of *ERBB2* amplification, a major biomarker for breast cancer, associated with the prescription of several new therapies targeting the HER2 protein. On cell lines, we demonstrated that the method allows not only mutation detection, as previously achieved in several microfluidic systems[4–7], but also a quantification of gene amplification, with a level of reproducibility comparable with that currently achieved on microslides, considered as the gold standard method. We also demonstrated, in a blind protocol on two clinical case studies, that our method retains its potential and quality of imaging when dealing with real clinical samples.

Regarding costs, our chip typically uses 10 to 20 times less DNA probes (the most expensive reagent). The chips themselves are made of COC, a low-cost and medically approved material amenable to mass microfabrication. Other important contributions to the cost of the assay involve the equipment needed to perform one experiment, as compared to conventional protocols. In our setup, a simple

syringe pump could be used to inject all the reagents sequentially and a calibrated hot plate can be enough to control the temperature inside the chip, and we believe that automation of the complete procedure can be easily achieved thus providing a further reduction of the total cost.

Last but not least, the reduction of volume consumption of our chips, as compared to current methods, also concerns the sample. It will thus allow performing more extensive genomic characterization on small samples. This is particularly timely for two reasons. First, the current evolution of oncology uncovers an unexpected complexity in the genetic landscape of cancers, and the number of genes to be screened for diagnosis is regularly increasing. Second, in a trend to minimize patient's risk and discomfort, diagnosis sampling is evolving towards minimally invasive methods, such as Fine Needle Aspirates (FNA)[31] , which provide samples in the order of tens to a hundred microliters. In our validation experiments, we used conservatively 20 µL of sample, a volume typically 10 times smaller than in conventional method, and we could indeed use routinely as little as 5 µL without significant degradation of the results. The method thus appears as an ideal companion for these promising therapeutic developments. More generally, this microfluidic approach opens the route to an expansion of the use of FISH in various applications including clinical diagnosis, an area in which it has not reached its full potential due in part to cost and complexity of implementation.

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Figures:

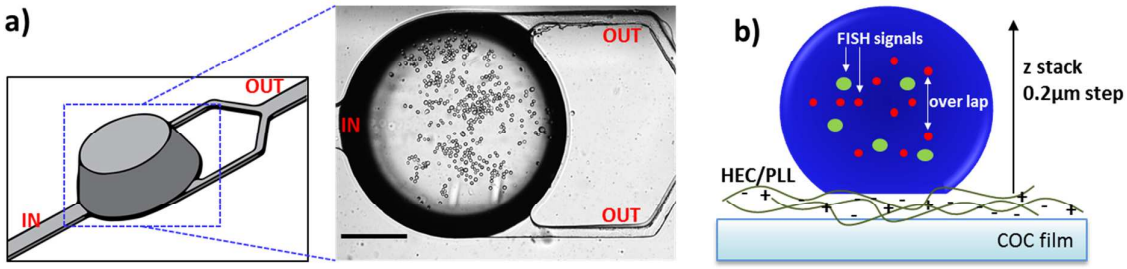

Figure 1 : a) Schematic view of the COC chip showing the"3D" chamber (height=0.38 mm, bottom diameter=1 mm) and the narrow microchannels (height=30 µm, width=60 µm) (left) and a micrograph showing the cells immobilized at the bottom of the chamber (right) at low magnification using bright field microscopy (scale bar=300 µm), b) Schematic representation of cells immobilized on the treated surface of the COC chip for FISH analysis.

Step on the FISH protocol		Glass slide		COC chip		
		Volumes (µL)	Incubation Time (min/T°)	Volumes (µL)	Incubation Time (min/T°)	Pressure (kPa)
1	Cell adhesion	200 (~2x10^5 cells)	O.N.[a] /37°C	20 (~2x10^4 cells)	30/R.T.[b]	1-4
2	1st washing	25, 000	2 /37 °C	200	-	10- 20
3	Enzymatic digestion	25, 000 [C]=0.05 µg/mL	7 /37 °C	100 [C]=1 µg/mL	7/37°C	10-20
4	2nd washing	(2 x) 25, 000	4 /37 °C	150	-	10-20
5	Fixation	25, 000	20 /5°C	100	20 /5°C	10-20
6	3rd washing	25, 000 25, 000	10/37°C 2/R.T.	(2 x) 200	10/37°C	35
7	Dehydration and drying	(3 x) 25, 000	(3 x) 2/R.T. 15/R.T.	**NO**	**NO**	**NO**
8	Probe injection and denaturation	10 [C]=1X	15/75°C	5 [C]=0.5 X	15/75°C	35
9	Hybridization		O.N. /37°C		O.N. /37°C	
10	Post-hybridization washing 1	(2 x) 25, 000	2/72°C	(2 x)100	4/72°C	30-35
11	Post-hybridization washing 2	(2 x) 25, 000	2/72°C	(2 x) 100	-	30-35
12	Dehydration and drying	(3 x) 25, 000	3 x 2/R.T 15/R.T.	**NO**	**NO**	**NO**
13	Counter staining	10	10/R.T.	5	10/R.T.	35

Table 1 : Steps of the FISH protocol on standard glass slide vs. COC chip, showing the reduction of volumes of reagents and changes in concentration of pepsin and probe solutions. No dehydration and drying steps are performed in the protocol on chip.

a:O.N.=Over night, b: R.T.= Room temperature

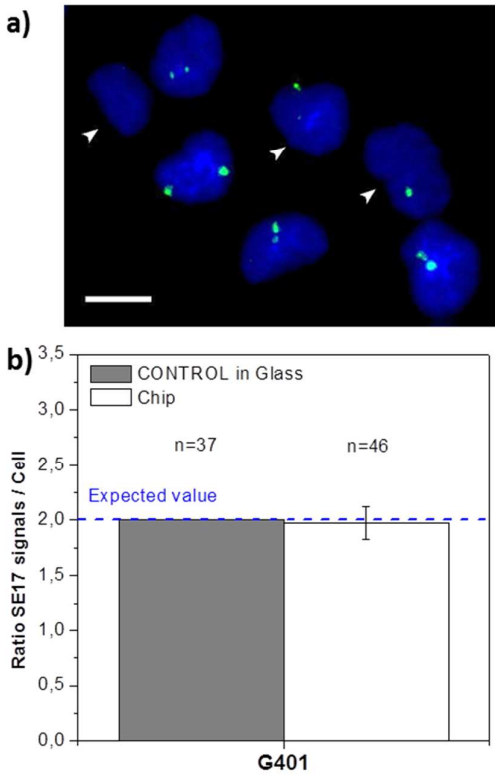

Figure 2 : G-401 cell lines after FISH with the SE17 centromeric probes. a) Fluorescence imaging of FISH results before optimization of the miniaturized protocol on COC chip. Several nuclei (white arrows) only present one or no signal for the SE17 centromeric probe, as a consequence of incomplete EtOH drying (scale bar=5 µm). b) Performance of the flow-through FISH protocol on chip after optimization, compared to the standard protocol on glass slide. Error bars show the Standard Deviation (S.D.)

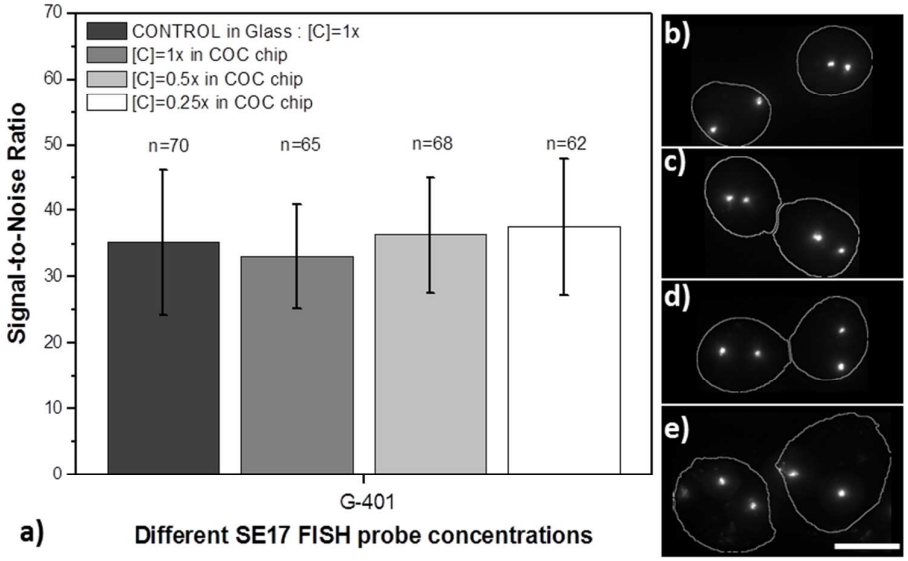

Figure 3 : a) Signal-to-Noise ratio measured on G-401 cell lines after FISH analysis of the signals from the SE17 centromeric probes on glass slides with the standard probe concentration (e: [C]=1x) and on chip with different probe concentrations (b:[C]=1x, c:[C]=0.5x, d:[C]=0.25x, scale bar=5 µm). SNR was measured as the ratio of the corrected maximum intensity of the peaks from FISH signals and the standard deviation of the intensity background coming from the cell nuclei. All measured cells contained 2 distinct SE17 signals. Error bars show the S.D.

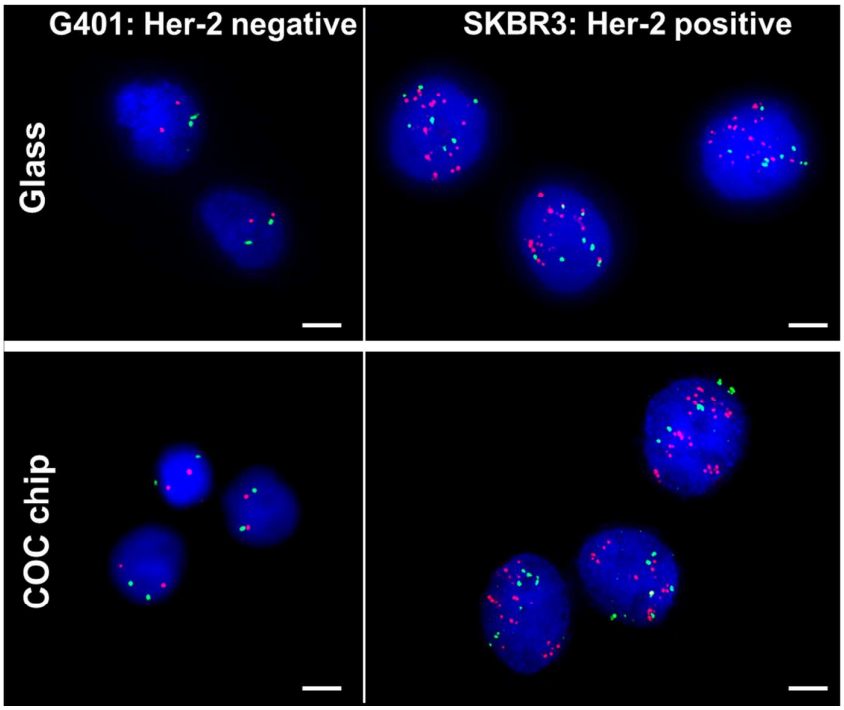

Figure 4 : High magnification fluorescence imaging of FISH results on glass slide (a, b) and on COC chip(c, d) for the HER2 typing of G-401(a, c) and SKBR-3 (b, d) cell lines (scale bar=2 µm)

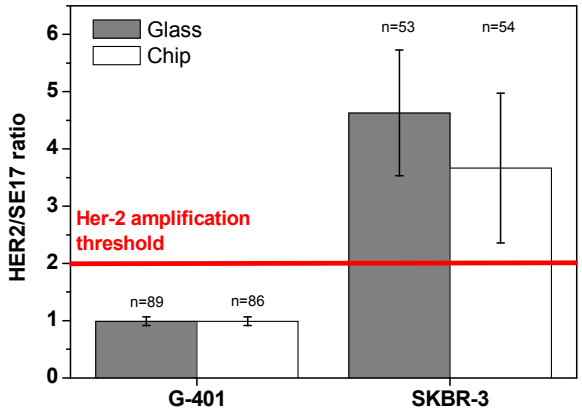

Figure 5 : FISH protocol validation on chip vs glass slides using two control cell lines with different karyotype and different HER2 status. Error bars show the S.D.

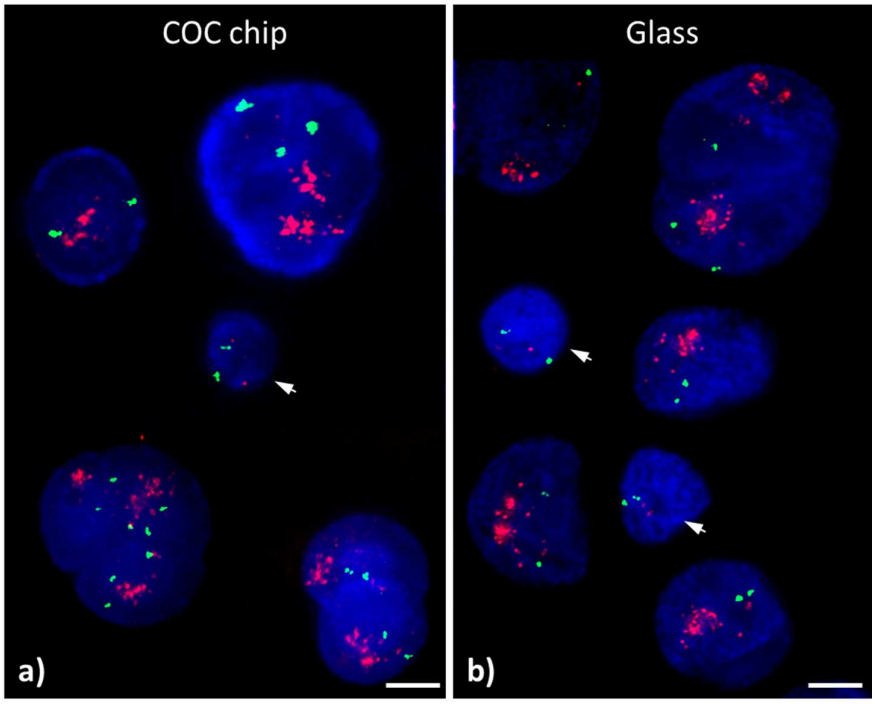

Figure 6 : FISH results for HER2 typing of cells from a pleural effusion from a breast cancer patient (Sample B) on COC chip (a) and on glass slide (b). White arrow shows possible leucocytes (scale bar=5 µm)