Microfluidics-assisted fluorescence *in situ* hybridization for advantageous human epidermal growth factor receptor 2 assessment in breast cancer

Huu Tuan Nguyen¹, Raphaël Trouillon¹, Seiya Matsuoka¹, Maryse Fiche², Laurence de Leval², Bettina Bisig² and Martin AM Gijs¹

Fluorescence *in situ* hybridization (FISH) is one of the recommended techniques for human epidermal growth factor receptor 2 (HER2) status assessment on cancer tissues. Here we develop microfluidics-assisted FISH (MA-FISH), in which hybridization of the FISH probes with their target DNA strands is obtained by applying square-wave oscillatory flows of diluted probe solutions in a thin microfluidic chamber of 5 μl volume. By optimizing the experimental parameters, MA-FISH decreases the consumption of the expensive probe solution by a factor 5 with respect to the standard technique, and reduces the hybridization time to 4 h, which is four times faster than in the standard protocol. To validate the method, we blindly conducted HER2 MA-FISH on 51 formalin-fixed paraffin-embedded tissue slides of 17 breast cancer samples, and compared the results with standard HER2 FISH testing. HER2 status classification was determined according to published guidelines, based on average number of *HER2* copies per cell and average *HER2*/CEP17 ratio. Excellent agreement was observed between the two methods, supporting the validity of MA-FISH and further promoting its short hybridization time and reduced reagent consumption.

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Fluorescence *in situ* hybridization (FISH) is a cytogenetic technique that targets a specific DNA or RNA location on a chromosome with a fluorophore-labeled oligonucleotide probe that is complementary to the target nucleic acid sequence.¹ In diagnostic and research laboratories, interphase FISH is widely used on formalin-fixed paraffin-embedded (FFPE) tissue sections of cancer samples, to detect numerical and structural chromosomal alterations such as aneuploidies, amplifications, deletions, and translocations.² However, its dissemination is impeded, among other factors such as its technical and analytical complexity, by the high cost of the probe and the long protocol times. Therefore, many studies focused on reducing the duration and reagent consumption of FISH tests through different approaches, including custom hybridization probes,^{3,4} electric field mixing,⁵ and modification of the hybridization buffer composition.⁶

In particular, microfluidic devices, where pL to μl volumes of fluid are manipulated, were used for implementing on-chip FISH on cells. Pioneering work was reported by Sieben *et al.*,⁷ who demonstrated that, by using recirculating hydrodynamic

flow or electrokinetic transport, it was possible to decrease by ~90% the reagent volume and to reduce the hybridization time to few hours for FISH staining on adherent cells. The same group also reported a fully automatic cell-based microfluidic FISH, which consumed ~5% of the standard used probe volume.⁸

Recently, for implementing rapid, economical, and multiplexed FISH for cell analysis, Huber *et al.* proposed scanning a vertically oriented capillary that creates a hydrodynamic flow of FISH probe on a small area (~0.096 mm²) of the cell slide surface.⁹ However, this technique can work only with fast hybridization centromere probes and has a small footprint due to the limited size of the capillary.

Although on-chip FISH analysis for immobilized cells is well-documented,^{9–16} few examples of on-chip FISH analysis of FFPE tissue have appeared in the literature, partially because of the difficulty of miniaturizing the set-up while preserving a large staining area.

About 15–20% of breast cancer patients have amplified and/or overexpressed human epidermal growth factor

¹Laboratory of Microsystems 2, École Polytechnique Fédérale de Lausanne EPFL, Lausanne, Switzerland and ²Institute of Pathology, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland

Correspondence: Professor MAM Gijs, PhD, EPFL, STI/IMT/Laboratory of Microsystems 2 (LMIS 2), BM 3128, Station 17, Lausanne 1015, Switzerland.

E-mail: martin.gijs@epfl.ch

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receptor 2 (*HER2*, also referred to as *ERBB2*) gene, which is associated with poor prognosis but predicts tumor response to *HER2*-targeted therapies, such as trastuzumab and other agents, which improve the patients' survival.^{17–20} Reliable assessment of *HER2* status is therefore of paramount importance and should be accomplished according to published guidelines.^{21,22} In a clinical perspective, immunohistochemistry (IHC) can be used for *HER2* detection, and is cheaper (~100–300 \$/test) and faster (2 h/test) than FISH (~300–800 \$/test, 2 days/test with an overnight incubation).^{18–20} However, standard IHC is largely qualitative, which may result in diagnostic ambiguities, whereas FISH is quantitative. In practice, in most pathology laboratories, *HER2* status of a newly diagnosed breast cancer is first assessed by IHC, equivocal IHC cases are then elucidated by FISH.^{21,22} In this context, on-chip FISH for analysis of clinical tissue slides was recently reported for detecting *HER2* amplification.²³ In this design, a removable polydimethylsiloxane (PDMS) chip was clamped against a glass slide containing a tissue or immobilized cells to create a chamber. Although this chip could automatize the FISH process and consumed only 2 μ l of probe solution, which is 1/5th of the classical probe volume per test, a 5 $\times$ 5 mm² tissue surface had to be used, which is about 20 times smaller than that used in the standard protocol. Also, no improvement of the hybridization time was reported. Nevertheless and despite the high economic impact, FISH on a chip for either cells or tissue was never clearly established in a pre-clinical study and its potential for routine diagnostic use not unambiguously demonstrated.

To address these challenges, we developed microfluidics-assisted FISH (MA-FISH), in which hybridization of the FISH probes with their target DNA strands is obtained by applying square-wave oscillatory flows of diluted probe solutions in a thin 16 $\times$ 16 mm² microfluidic chamber of 5 μ l volume. The microfluidic system is based on a previously reported microfluidic chip used for the accurate IHC staining of breast carcinomas.^{24,25} The study consisted in two phases: optimization of MA-FISH experimental conditions, followed by validation of the optimized parameters by comparing MA-FISH with the standard FISH technique. Using the optimization parameter set, we show that MA-FISH can be performed on a tissue surface as large as 16 $\times$ 16 mm² and uses only 1 μ l of the standard probe solution locus-specific identifier *HER2*/CEP17 (PathVysion Kit, ABBOTT, IL, USA), which can be diluted, thus further decreasing the consumption of the expensive hybridization probe solution per unit area of tissue by a factor 5. Moreover, the total duration of a test is decreased from 2 days to 1 day, mostly because of the short 4-h hybridization. To the best of our knowledge, MA-FISH is the first example of tissue analysis offering both cost and time reduction. In the validation phase, we conducted *HER2* MA-FISH on 51 FFPE tissue slides of 17 breast cancer samples, and compared the results with those obtained from standard *HER2* FISH testing. *HER2* status classification by

MA-FISH was in excellent agreement with standard methods, yielding Pearson's correlation coefficients of 0.98 and 0.95 between the number of *HER2* copies per cell (*HER2*/cell) and the average *HER2* to CEP17 ratio (*HER2*/CEP17 ratio), respectively, thus proving the applicability of MA-FISH for *HER2* status assessment on breast cancer tissues.

MATERIALS AND METHODS

Chemicals and Materials

All the reactants were purchased from Sigma, unless stated otherwise, and were used as received without further purification. All the solutions in this work were made using 18 M Ω /cm water obtained from a Millipore purification system. For the FISH staining, the PathVysion *HER-2* DNA Probe Kit (Abbott Molecular, IL, USA) was used and consisted of two differentially labeled probes focused at two different targets. The *HER2* gene locus (17q11.2-q12) is targeted by a 190 kb SpectrumOrange-labeled probe, which comprises several complementary DNA segments of the specific gene, and in our analysis was assigned a red color. The centromeric regions of chromosome 17 (CEN17) (17p11.1-q11.1) are localized by the 5.4 Kb SpectrumGreen-labeled Chromosome Enumeration Probe 17 (CEP17) DNA probe, which comprises complementary DNA segments of the alpha-satellite region located at the centromere of chromosome 17, and in our analysis was assigned a green color.

Sample Selection and Tissue Slide Preparation

Seventeen FFPE invasive breast carcinoma samples, diagnosed between 2012 and 2015, were retrieved from the archives of the Institute of Pathology from the Vaud University Hospital (CHUV, Lausanne, Switzerland), selected to span a wide range of different *HER2* copy numbers per cell, and including six negative, five positive, and six equivocal *HER2* cases according to 2013 ASCO/CAP guidelines.²¹ For each sample, 4 μ m thick tissue sections were cut from a representative FFPE block and mounted on Super Frost Plus slides (Menzel-Glaser, Germany). The invasive component of the carcinoma was located by a pathologist on adjacent hematoxylin and eosin stained slides. As part of an ethical convention (BB514/2012) with the Ethical Commission of Clinical Research of the state of Vaud, all tissues used in this study have been anonymized, codified, and all patients have not expressed any objection to the use of their tissue.

Microfluidic Chip and Set-Up

The microfluidic chip (see Figure 1a) was micro-fabricated using the protocol illustrated in Supplementary Figure S1 in the Supplementary Information. The microfabrication process flow was adapted from ref. 24. Briefly, it consisted of a bonded Pyrex-Si stack: the Pyrex side (Figure 1ai) showed 'tree'-like channels allowing a homogenous distribution of liquid for uniform staining of the tissue; the liquid was channelized *via* feedthrough holes to the Si side of the stack (Figure 1aii) that later formed, using mechanical clamping *via*

Figure 1 Design of the microfluidic system. (a) The microfluidic chip consists of a bonded Pyrex-Si stack: (ai) view on the Pyrex side of the stack showing ‘tree’-like channels etched in Si allowing a homogenous distribution of the liquid for uniform staining of the tissue; the flow is channelized via feedthrough holes, e.g., the array encircled in white, to (aai) the Si chip face that later will form the microfluidic chamber, together with the spacer strips, PDMS o-ring, and the tissue slide. (b) Exploded view of the set-up. A microscope slide carrying a tissue section is mechanically clamped against a microfluidic chip and thereby forms the bottom of a thin $16 \times 16 \text{ mm}^2$ size chamber. Two strips of Al foil serve as spacers controlling the chamber height to $20 \mu\text{m}$, whereas a PDMS o-ring hermetically seals the chamber and the fluidic circuit, which are placed in a thermostatic copper holder. (c) View of the upside-down assembly of the set-up of b on a hot plate, which itself is to be positioned in a thermally insulating polystyrene chamber afterwards. (d) Three-dimensional representation of the channels and the $5 \mu\text{l}$ microfluidic chamber, in which square-wave oscillatory flow cycles are applied during the MA-FISH protocol. (di) and (dii) show the extreme situations of a cycle; the total staining solution volume (indicated in blue) is only $10 \mu\text{l}$ and is flanked by perfluorinated oil that fills the rest of the channels.

screws, the microfluidic chamber together with the spacer strips, PDMS o-ring, and the tissue slide (see Figure 1b). A controlled screwing force during assembly was provided by a dynamometric screw driver (TorqueVario-S, Wiha Werkzeuge GmbH, Germany) for reproducibility. Two $20 \mu\text{m}$ thick aluminum strips served as spacers for fixing the chamber height at $20 \mu\text{m}$, whereas a $350 \mu\text{m}$ thick PDMS o-ring (Shielding Solutions, Essex, UK) was inserted in a $275 \mu\text{m}$ deep notch to hermetically seal the chamber. A rubber o-ring distributed the clamping force homogeneously, and also contributed to a better thermal insulation of the chamber. The assembly of these elements was placed in a copper holder (Figure 1b and c), the temperature of which was controlled by placing it on a hot plate (Scilogex MS-H280-Pro, Thomas Scientific, NJ, USA). Note that the assembly of Figure 1b was presented upside-down for better visualization. Thermal stabilization was realized via readout of an external thermometer probe (Fluke 54ii, Fluke, WA, USA) that was in contact with the microscope slide. During operation, the copper holder was also covered by a polystyrene foam-based thermal insulation chamber. Finally, the copper holder was interfaced via commercial microfluidic fittings (see Figure 1c) to two automated syringe pumps (neMESYS, Cetoni GmbH, Germany).

Standard FISH Protocol

The standard FISH protocol was adopted from the standard practice used at the Institute of Pathology and repeated for the purpose of the study. The detailed protocol is described in Supplementary Information and a typical fluorescence image is shown in Supplementary Figure S2.

MA-FISH Protocol

The de-paraffinization, pre-treatment, protein digestion, post-fixation, and washing steps were identical to that of the standard protocol and were done off-chip. However, the denaturation and hybridization steps were performed differently from the standard FISH protocol. The details of the protocol and the probe dilution method for MA-FISH protocols are provided in Supplementary Information, the dilution proportion was similar to that of ref. 26. Briefly, $10 \mu\text{l}$ of diluted probe solution were loaded in a syringe pump system and injected to the surface of the tissue via the microfluidic chip. Then a push-pull injection pattern, characterized by its frequency f , was applied to the fluid for inducing a displacement of $5 \mu\text{l}$ of probe solution over the sample (Figure 1di and dii). The MA-FISH protocol was optimized by studying series of different values of different variables, such as probe dilution, hybridization time, and flow rate (*vide infra*).

Image Acquisition

The same image acquisition and scoring method were applied for both MA-FISH and standard FISH. A fluorescence microscope (Carl Zeiss Axio Imager M2m) was used for the imaging. Filter set, objective, and camera settings are all detailed in Supplementary Information. To allow high-quality imaging, z-stacks were obtained for n layers, with $n = h_{\text{tissue}}/\Delta z$, where h_{tissue} is the tissue thickness ($\sim 4 \mu\text{m}$) and Δz is the axial increment step (Figure 2a). This parameter could be tuned depending on the purpose of the analysis: for counting signals within a stack, Δz was $0.5 \mu\text{m}$. For a three-dimensional image reconstruction for a graphical presentation, Δz was fixed at $0.2 \mu\text{m}$. The explanation of this choice is detailed in Supplementary Information. With these parameters, three-channel (blue, green, and red) z-stacks were recorded for the regions of interest of the slide, each corresponding to a volume of $300 \times 400 \times h_{\text{tissue}} \mu\text{m}^3$ of tissue. To facilitate the acquisition, each plane of the xy image was divided into a 3×3 mosaic (Figure 2b, zoom to a region of interest in Figure 2c), for which thereafter the whole three-dimensional volume was reconstructed. The $0.2 \mu\text{m}$ step z-stack images were de-convoluted with the Huygens software (HRM software, AutoDeblur, BitPlane, Switzerland), followed by a three-dimensional reconstruction with the software IMARIS (BitPlane, Switzerland). The fluorescence signal, for each channel, was then projected on the xy plane. Results for a HER2-negative case and a HER2-positive case were selected for the graphical representation in Figure 2d and e, respectively. As an internal control of the technique, smaller non-cancerous nuclei with normal HER2 status, adjacent to HER2-positive cancer cells, are shown in Figure 2f.

Image Analysis

An unbiased, quantitative, and automated signal analysis based on an image processing pipeline, developed using the open-access software Cell Profiler (Broad Institute)²⁷ is detailed in Supplementary Information and illustrated in Supplementary Figure S3. The purpose of this analysis is to extract the diameter and intensities of each dot associated to the FISH signal, and allow for the comparison of different experimental conditions. Then, a contrast function, defined as $(I_{\text{sg}} - I_{\text{bg}})/(I_{\text{sg}} + I_{\text{bg}})$, with I_{sg} and I_{bg} the average intensities of the signals associated to either the green or red dot and the background, respectively, is computed. The numerical results for the different parameters were compared using a one-way analysis of variance (ANOVA) test followed by a *post-hoc* Tukey's comparison test. The latter helps justifying our graphical observation, *vide infra*. The results were presented as mean $\pm$ standard deviation (s.d.), obtained by averaging diameter and contrast from nine images in the selected 3×3 mosaic picture. Prism software (Graphpad Software, CA, USA) was used to plot the results.

MA-FISH Scoring

To assess MA-FISH scores, z-stacks ($\Delta z = 0.5 \mu\text{m}$) were obtained as detailed above, for three independent invasive locations of the sample. Axiovision software (Carl Zeiss, Germany) was used to inspect each layer of the z-stack. The scoring method and classification criteria, based on the 2013 ASCO/CAP guidelines,²¹ are detailed in Supplementary Information. According to these guidelines, two scores, the average HER2 copy number/cell and the HER2/CEP17 ratio, should be reported for HER2 status assessment in clinical practice.

Validation Method

To test the diagnostic power of the MA-FISH technique, as optimized in the parameter study sections (*vide infra*), we performed a series of tests on a set of 17 breast cancer samples chosen to represent a wide spectrum of different HER2 copy numbers per cell and different HER2 statuses. During the initial HER2 assessment performed at the Institute of Pathology using the standard off-chip protocol, six cases were classified as HER2-negative, six were equivocal, and five were positive. However, to ensure a maximal consistency in the comparison of the MA-FISH technique with the standard protocol, the standard routine technique was repeated in the research laboratory to ensure that the use of other reactants, counting operators, and microscopic equipment, as well as the storage time of the biopsy sample in the tissue bank, did not introduce discrepancies with respect to the standard benchmark. To perform this study, several adjacent sections were used for each tissue sample. One section was processed according to the standard FISH protocol repeated in our laboratory, to provide the control data set representing the current state of the art, further referred to as 'in-house standard FISH'. In parallel, three adjacent sections were processed with the MA-FISH system to address the question of technical reproducibility. Each slide was then blindly scored for (i) the count of HER2/cell and (ii) the HER2/CEP17 ratio. The counting routine used here based on z-stacks with $\Delta z = 0.5 \mu\text{m}$. For each slide, considering the biological variability issue, the average number of HER2/cell and the overall HER2/CEP17 ratio of a cluster of 20 cells were obtained for three clusters, corresponding to three separate locations on the tumor area. For the HER2 status assessment, the 2013 ASCO/CAP recommendations were used.²¹ Briefly, the average HER2 copy number/cell and the HER2/CEP17 ratio were obtained from three MA-FISH slides from the same patient. These scores were then averaged and compared with the cutoff values, for each parameter, reported in the 2013 ASCO/CAP recommendations to deduce the HER2 status of the patient. The variance among the three position scores for each slide was also computed, then averaged. The s. d. represents the scoring difference between clusters, and is different from the one obtained by pooling the average numbers of HER2/cell and the HER2/CEP17 ratio of the three cases. The latter would incorporate slide-to-slide variability

Figure 2 FISH signal presentation. (a) Evaluation of the MA-FISH signal is based on a set of 3×3 mosaic z-stack images (typically 21 layers, $\Delta z = 0.2 \mu\text{m}$), recorded at different positions of the tumor area after the staining process. The recorded images span a tissue volume of $400 \times 300 \times 4 \mu\text{m}^3$. (b) The nine images of one layer of the z-stack. (c) Zoom on a region of interest of b, from which the FISH signal can be acquired. (d) Projected image of the three-dimensional region of interest in a single plane, following deconvolution of all layers of the z-stack (HRM software, Scientific Volume Imaging B.V., Netherlands) and three-dimensional reconstruction (IMARIS software, Bitplane, Switzerland) of the latter. This is a HER2-negative case (ie, not amplified), which shows one to two copies of the *HER2* gene (corresponding to the red signals) per nucleus in the cancerous cells (their large nuclei can be appreciated thanks to the blue DAPI counterstain), and an approximately equivalent number of copies of centromere 17 (CEP17, corresponding to the green signals). (e) Projected image of a HER2-positive case (ie, amplified), in which the cancer cells have large nuclei containing >6 *HER2* copies (red signals) per nucleus, whereas the number of CEP17 (green signals) is not increased, yielding a *HER2*/CEP17 ratio >2 . (f) The same case as in e, but focusing on a region in the tissue of non-cancerous cells that have smaller nuclei and show no amplification of the *HER2* gene (internal negative control).

effects, which is represented by the coefficient of variation of triplicates, obtained by normalizing the s.d. of the triplicates to their mean. On the other hand, the former s.d. only

describes the signal variability in the slide and was taken as a more reliable marker of data dispersion due to biological variability.

RESULTS

Considerations on the System Variables

To facilitate the analysis and guarantee an optimal diagnostic power, the FISH staining protocol should preserve the morphology of the tissue, result in a homogenous staining of nuclei with low auto-fluorescence from background materials, and feature well-defined, bright, DNA-specific FISH signals inside the nuclei. In a standard set-up, DNA hybridization can be tuned by several experimental factors, such as the denaturation (T_d) and hybridization (T_h) temperatures, the denaturation (t_d) and hybridization (t_h) times, the composition of the buffers, the probe concentration C , etc. However, in the case where FISH is implemented in a microfluidic system, the flow rate Q and the microfluidic chamber height h will also all have an effect on the outcome of the test. Finally, the probe density d , ie, the ratio of the total volume of undiluted probe solution used in the protocol V to the stained surface area of the section S is also introduced. The probe density can be seen as the cost, in terms of volume of undiluted probe solution, of the technique per unit of tissue surface, and has the dimension of a distance. This figure-of-merit was used as a way to allow up-front comparison of different experimental techniques (for instance standard vs MA-FISH) by normalizing the amount of probe used for each technique to the surface area of the target.

From these considerations, the analysis of the effects of the experimental parameters affecting the DNA hybridization step in a MA-FISH protocol is a multi-parameter variational problem. However, some of these parameters are fixed according to the specificities of the standard protocol, such as T_d , T_h , or the buffer compositions, and others are not independent variables, such as d , and can be computed from other parameters (V , S , and C). Furthermore, and to keep the dead volume to a minimum, h should be kept as low as technically possible. Therefore, to ensure that a total volume of probe solution could be used for each experiment, h was fixed at $20\ \mu\text{m}$ and was not modified in this work. Finally, only three parameters were varied in the following analysis: t_h , C , and Q . The other independent variables (T_d , T_h , t_d , V , S , etc) were left unchanged throughout the experiments. For the off-chip standard protocol, a d value of $\sim 21\ \mu\text{m}$ ($\sim 0.021\ \mu\text{l}/\text{mm}^2$) had been used, following the recommendations of the manufacturer. The parameters t_h , C , and Q were optimized. To ensure that the results for the different conditions, for a given parameter, were comparable, only adjacent slides from the same tissue block were used. As the tested material (human tissue sections) was limited, only one comparison test was performed for each condition for each parameter (t_h , C , and Q). Furthermore, only conditions that were *a priori* expected to improve the technique (shorter times, diluted probes, etc) and were also expected to not compromise dramatically the signal intensity were selected for this optimization.

Optimization of the Dilution Factor

To study the effect of C on the MA-FISH capabilities, three adjacent sections, obtained from the same tumor, were processed on-chip using the MA-FISH protocol with three different dilution factors ($5\times$, $10\times$, and $20\times$) of a standard commercial probe solution (PathVysion HER-2 DNA Probe Kit), with $Q=1\ \text{nl/s}$, $f=10^{-4}\ \text{Hz}$, and $t_h=4\ \text{h}$. These three sections were processed during three successive days and imaged with identical exposition conditions on the same day. A fourth adjacent section from the same sample was processed and imaged with the standard FISH method, which used a non-diluted probe, and served as a control. As detailed above, several numerical variables (signal diameter and contrast) were obtained to characterize the quality of the FISH staining, and are presented in Figure 3. From the data shown in Figure 3ai, the $20\times$ dilution configuration resulted in smaller diameter of the red and green signals than the $10\times$ dilution ($P<0.0001$ for red and <0.001 for green) and the $5\times$ dilution ($P<0.0001$ for red and <0.0005 for green) (Figure 3ai). An increased dilution yielded smaller green signal contrast for the $20\times$ dilution than the $10\times$ and $5\times$ dilution ($P<0.0001$ for both), but did not significantly affect the red signal contrast (Figure 3a_{ii}). All ANOVA and *post-hoc* P -values are represented in the Supplementary Information and Supplementary Table S1. Finally, the average number of *HER2*/cell and the *HER2*/CEP17 ratio in three clusters of 20 cells were also computed. As the sections were obtained from adjacent positions from the same tumor, similar scores are to be expected. Indeed, the $10\times$ and $5\times$ dilution yielded similar results as the standard FISH method (Figure 3a_{iii}), but a lower number of *HER2*/cell was found for the $20\times$ dilution. Overall, these results indicated that the $10\times$ dilution factor for the PathVysion probe was the preferable one, as it resulted for our MA-FISH set-up into comparable results to the standard technique.

Optimization of the Hybridization Time t_h

As above, the effect of t_h was investigated by incubating three adjacent slides originating from the same tumor for 2, 4, and 8 h, respectively, in a $10\times$ diluted probe solution with $Q=1\ \text{nl/s}$ and $f=10^{-4}\ \text{Hz}$ (see Figure 3b). The sample corresponding to $t_h=4\ \text{h}$ is the same as the one used in the dilution factor analysis for a $10\times$ dilution, but was analyzed again alongside the other samples. The 4-h hybridization yielded the same type of response as for $t_h=8\ \text{h}$, as suggested from the values of the signal diameter in the red and green channels, but a noticeable degradation of the quality of the FISH staining was noticed for the 2-h hybridization. Indeed, from the data shown in Figure 3b, the 2-h hybridization configuration resulted in smaller red signals ($P<0.0001$) and green signals than the 4 h ($P=0.001$) (Figure 3bi). It also yielded weaker green and red signal contrast than the 4 h ($P<0.005$) (Figure 3b_{ii}). The signal count on the 2-h hybridization MA-FISH slide resulted in a lower number of *HER2*/cell and lower

Figure 3 Experimental parameter study of the MA-FISH protocol: influence of probe dilution, hybridization time, and flow rate. **(a)** Optimization of the probe dilution. Four adjacent slides originating from the same tumor were incubated on-chip during 4 h with three different dilutions (5×, 10×, and 20×) of a standard commercial probe solution (PathVysion, Abbott), or off-chip using the standard protocol. The on-chip square-wave oscillatory flow was applied with an amplitude of 1 nl/s at a frequency of 10^{-4} Hz. The green bars were obtained by averaging the green signal diameter and contrast (defined as $(I_{sg} - I_{bg}) / (I_{sg} + I_{bg})$, with I_{sg} and I_{bg} the signal and the background intensity, respectively) originating from the CEP17 and the red bars were obtained by averaging the red signal diameter and contrast originating from the HER2 probes from a mosaic image composed of nine tiles ($\times 63$ microscope objective) and using an image processing program (Cell Profiler). The last two bars correspond to standard off-chip overnight hybridization conditions with an undiluted solution. The different panels represent **(ai)** the average diameter of a signal, **(a ii)** the average contrast of a signal, and **(a iii)** the average count of red signals (HER2) per nucleus of a cell ($HER2/cell$) for the three probe dilutions and the standard, obtained from three different positions for each slide (20 cells evaluated for each position, full line, left axis) and the $HER2/CEP17$ ratio (dashed line, right axis). **(b)** Optimization of the hybridization time. Four adjacent slides originating from the same tumor were incubated on-chip with a 10× diluted probe with a flow rate of 1 nl/s at a frequency of 10^{-4} Hz, using three different hybridization times (8, 4, and 2 h), respectively. The last two bars in each graph correspond to standard off-chip overnight hybridization conditions. **(bi)** The average diameter of a signal. **(b ii)** The average contrast of a signal. **(b iii)** $HER2/cell$ number for the three hybridization times and the standard, obtained from three different positions for each slide (20 cells evaluated for each position, full line, left axis) and the $HER2/CEP17$ ratio (dashed line, right axis). **(c)** Optimization of the flow rate. Five adjacent slides originating from the same tumor were incubated on-chip during 4 h using a 10× diluted probe, using five different flow rates of the on-chip square-wave oscillatory flow (0, 1, 10, 100, and 1000 nl/s) corresponding to push-pull cycle frequency $f = 0, 10^{-4}, 10^{-3}, 10^{-2}$, and 10^{-1} Hz, respectively. **(ci)** The average diameter of a signal. **(c ii)** The average contrast of a signal. **(c iii)** $HER2/cell$ number for the five flow rate conditions and the standard, obtained from three different positions for each slide (20 cells evaluated for each position, full line left axis) and the $HER2/CEP17$ ratio (dashed line, right axis).

$HER2/CEP17$ ratio comparing to the use of 4- and 8-h hybridization times ($P < 0.0005$ for $HER2/cell$ number and < 0.001 for the ratio), the latter times resulting in the same count than the standard technique (Figure 3biii). From this analysis, an optimized hybridization time of 4 h was chosen, hereby resulting in a significant shortening of the experimental duration, in comparison to the standard protocol (16-h hybridization). The green signal in an 8-h hybridization MA-FISH test showed fluorescence intensity reduction due to photo-bleaching, possibly because of air exposure, which however did not affect the signal count. It proved to be difficult to decrease the hybridization time below 4 h, since probe diffusion in the tissue and small DNA hybridization rate in formamide solution require sufficient incubation time.⁶

Optimization of the Flow Rate Q

To find an optimal Q for MA-FISH, five adjacent sections originating from the same tumor were incubated on-chip during 4 h in a 10× diluted probe, using five different Q for the on-chip square-wave oscillatory flow profile ($Q = 0, 1, 10, 100$, and 1000 nl/s). The characteristic parameters for each sample were computed and are reported in Figure 3c. The $Q = 1$ nl/s configuration resulted in the largest red signal diameter with respect to the other flow rates used, while there is a noticeable variation of the green signal diameter as function of Q, which is however not critical for interpretation, due to the larger size and high intensity of a green signal that represents the centromere-specific probe (Figure 3ci and Supplementary Table S1). The $Q = 1$ nl/s configuration resulted also in the largest contrast for the red signals with

Figure 4 Average number of *HER2*/cell and *HER2*/CEP17 ratio results from MA-FISH tests, in the full domain (left image) and in the sub-domain of *HER2*/cell number ≤ 6 and *HER2*/CEP17 ≤ 2 (right image). The test results that were obtained from slides of the same patient are represented by the same symbol in the graph. The dotted lines represent the cutoff values for *HER2* status classification according to 2013 ASCO/CAP guidelines. In the right panel, some borderline cases, laying close to the dotted lines representing *HER2*/cell = 4 and = 6, are more likely to result in different clinical classifications, hence accounting largely for the assessment discrepancies discussed in the text. The total number of tissue slides analyzed here was $N = 51$ (17 tissues samples, analyzed in triplicates).

respect to the other flow rates used, whereas variation in the green signal contrast as function of Q again is not critical for interpretation (Figure 3cii and Supplementary Table S1). The results for the number of *HER2*/cell and the *HER2*/CEP17 ratio (Figure 3ciii) did not allow for clearly establishing which Q led to the best results, largely because of the signal variation induced in this specific sample by a high level of genetic heterogeneity (Supplementary Table S1). The variation induced by the heterogeneity depends on the position of the clusters studied, and has a more important impact on the experimental output than the variation due to change in flow rate. Therefore, no significant difference between the flow rate conditions was found.²⁸

Summarizing, applying a flow of probe over the sample improves the reactant delivery with respect to the delivery by diffusion only and reduces depletion effects of the probe inside the chamber, as present with the quiescent flow condition, in which the tissue has consumed all local probes. On the other hand, if the flow rate is too high, it can affect the probe-target combination. It was demonstrated indeed that hybridization between target DNA and immobilized DNA on a surface of a DNA microarray could be impaired under a strong flow rate.²⁹ Therefore, DNA hybridization on a tissue section can also be affected by a strong flow, probably because of the presence of shear stresses near the tissue surface that hinder the hybridization events. As a consequence, we have chosen to use $Q = 1$ nl/s for the final MA-FISH protocol. In conclusion, the final MA-FISH parameter set, chosen from the optimization study, are $C = 10\times$ dilution, $t_h = 4$ h, and $Q = 1$ nl/s.

Technical Comparison between MA-FISH and Standard-Like Coverslip Technique but Using the same $10\times$ Dilution and 4 h Hybridization Time

We compared the MA-FISH technique with a standard-like coverslip-based protocol, in which we exposed a slide to a $10\times$ diluted probe (standard = $1\times$) and used a 4-h

hybridization time (standard = hybridization overnight, ie, 16 h). The probe density, presenting the probe solution used per footprint, was also kept the same as for MA-FISH ($d = 3.9\ \mu\text{m}$). This experiment was designed to show the signal enhancement granted by the hydrodynamic method. Seventeen sections from 17 different tissue blocs were processed with the coverslip method, were imaged by the same protocol than in MA-FISH slides and the resulting *HER2* status was also compared with the in-house standard FISH and reported in the Supplementary Information and Supplementary Table S3. As shown in Supplementary Figure S5 in the Supplementary Information, the MA-FISH results in much stronger signals compared with the coverslip-based technique, which showed a quasi-inexistent signal, even though the amount of probe introduced in the system and the image acquisition and processing were the same. Among them, 41% (7/17 cases) gave even no specific red signal for the scoring process. The reason of this difference is expected to arise from the constriction of the diffusion layer near the slide surface under hydrodynamic conditions, thus increasing the flux of probe toward the tissue and accelerating the hybridization. This confirmed the critical importance of the hydrodynamic conditions in improving the FISH signal.

Comparison of *HER2*/cell, *HER2*/CEP17 Ratio, and *HER2* Status between MA-FISH and Standard FISH Protocol

The final MA-FISH conditions using the optimized parameters described above were tested on 17 breast cancer samples, and the *HER2* results were compared with the ones obtained with the standard protocol. The reproducibility of MA-FISH shown by the consistency of the average number of *HER2*/cell and the average *HER2*/CEP17 ratio of triplicate slides are shown in Figure 4. A good reproducibility of the MA-FISH results was demonstrated as the coefficient of variation among triplicates was 11.2% for the *HER2*/cell number (95% confidence interval (CI), 8.4–14%) and 10.6% for *HER2*/CEP17 ratio (95% CI, 7.5–13.7%).

Figure 5 Comparison of the MA-FISH with the in-house standard FISH protocol. Comparison between the results obtained for the MA-FISH and standard technique for (a) *HER2*/cell number in the full domain (left image) and in the 0–6 sub-domain (right image) and (b) the *HER2*/CEP17 ratio in the full domain (left image) and in the 0–2 sub-domain (right image). The results shown here correspond to 17 independent cases. The data represented for each point were the mean scores of three MA-FISH adjacent sections vs the mean scores of an in-house standard FISH adjacent section, obtained from the same tissue block. The vertical error bars represent the s.d., with respect to the average of each specific slide, defined as the square root of the average of the variances obtained for each slide of the triplicates. The horizontal error bars are the s.d. of the scores among three positions in standard FISH slides. The dotted lines represent the cutoff values for *HER2* status assessment according to 2013 ASCO/CAP guidelines. The linear fits show a good correlation of the FISH results (*HER2*/cell number and *HER2*/CEP17 ratio) between the MA-FISH and the standard FISH (Pearson's correlation coefficients were 0.98 and 0.95, respectively).

The correlation between MA-FISH and in-house standard FISH scores with respect to the number of *HER2*/cell and the *HER2*/CEP17 ratio obtained from 17 patients is shown in Figure 5a and b, respectively. The numerical data are reported in Supplementary Table S2. The results of Figure 5 demonstrate Pearson correlation coefficients between the MA-FISH and standard protocol data sets of $r=0.98$ (number of *HER2*/cell) and $r=0.95$ (*HER2*/CEP17 ratio), respectively. Also a linear regression was computed for these data sets, with regression coefficients $\beta=0.78$ ($R^2=0.97$) and $\beta=0.93$ ($R^2=0.89$), respectively. Although the first regression coefficient may appear low, it mainly results from the lower fluorescence intensity for the highly amplified *HER2* cases due to the use of diluted probes. However, in these cases, the diagnostic outcome of the MA-FISH technique with respect to the standard protocol does not change (*vide infra*).

To compare the *HER2* statuses obtained from MA-FISH with the ones from the standard FISH, the concordance between the average results of 17 MA-FISH triplicates from 17 independent tissue biopsies and standard FISH test results is reported in Table 1. Table 1 also indicates that some of the borderline positive or negative slides, according to the

standard test, became equivocal with the MA-FISH processing, and vice versa. The likeliest explanation is that the biological variability is here critical, as most of the slides were initially scored as equivocal, thus maximizing the risk for slide-to-slide variations (Figure 4, right image). Nevertheless, no positive case was classified as negative and vice versa, which ruled out the risk for false positive or false negative *HER2* status classification.

DISCUSSION

The concordance between a FISH result and another method, such as IHC, or between two FISH methods was studied in the literature, solely based on the comparison of the *HER2*/CEP17 ratio, taking a cutoff value for the ratio of 2 or 2.2.^{30–33} A general strategy for comparing clinical outcomes involving two techniques makes use of Cohen's Kappa K, which is a parameter quantifying the agreement between two sets of qualitative ratings.³⁴ This analysis was applied to our MA-FISH and standard FISH data sets. By setting the *HER2*/CEP17 ratio cutoff at 2 and using it as a single criterion to separate the positive and negative FISH populations (ie, there is no equivocal class), the concordance

Table 1 Concordance table

	In-house standard FISH protocol			Sum
	Negative	Equivocal	Positive	
MA-FISH protocol				
Negative	8	1	0	9
Equivocal	1	2	1	4
Positive	0	0	4	4
Sum	9	3	5	17

Comparison of the HER2 classification results of the microfluidics-assisted fluorescence *in situ* hybridization (MA-FISH) protocol and the in-house standard FISH protocol, based on the HER2 signal counting obtained from z-stacks of images. HER2 classification into three different groups (negative, equivocal, and positive), was obtained using 2013 ASCO/CAP recommendations, see Supplementary Information for details. The total number of breast cancer cases analyzed here was $N=17$. The MA-FISH statuses were obtained from the average results of triplicates, whereas the in-house standard FISH were from a single slide. No major classification disagreement (from negative to positive and vice versa) was found.

between the MA-FISH and in-house standard FISH results is evaluated with a score $K=1.00$ (100% concordance) (see Supplementary Table S4). On the other hand, if the assessment is based on three classes described for the number of *HER2* copies per cell (negative, positive, and equivocal) in the 2013 ASCO/CAP recommendations,²¹ the resulting Cohen's Kappa K is 0.71 (95% CI, 0.41–1.00), still showing a good concordance of MA-FISH and in-house standard FISH clinical results.³⁴ A similar analysis comparing the MA-FISH results to the initial clinical HER2 assessments as obtained at the Institute of Pathology, showed that the results are highly consistent with the clinical standard methods (see Supplementary Information and Supplementary Tables S2).

The roughness of the tissue was considered as a possible source of inaccuracy by inducing different diffusion pathways for different tissue areas, resulting in non-uniform staining. However, the hydrodynamic flow was applied in a cycling fashion and helped mixing the probe inside the chamber, thus avoiding local depletions. Moreover, the diffusion distance of probe during the hybridization time of 4 h is approximated by $x = 2\sqrt{Dt} = 0.88\text{mm}$, with $D = 1.33 \times 10^{-7} \text{ cm}^2/\text{s}$.³⁵ The effect of this thickness variation, expected to be at most 10 nm,²⁴ was therefore assessed as negligible. It was also investigated as a possible source of inaccuracy for FISH image readout. Here the variation of the tissue thickness should be avoided because it might lead to a change of the average HER2 signals per cell, one of the two recommended scores for HER2 classification.²¹ However, once again, comparing to the tissue thickness of 4 μm , the very limited roughness expected here should not dramatically alter the validity of the analysis.

From a technical standpoint, this study showed that MA-FISH is an advantageous, reliable technique to assess HER2 status on FFPE breast cancer tissue. On the one hand, by recirculating a diluted probe solution on the surface of the

tissue during the hybridization time, the use of expensive probe can be optimized and the cost of a FISH experiment drastically reduced. Comparing to a standard FISH protocol and considering the tissue surface stained, MA-FISH reduces the cost per footprint per test by a factor of 5. Moreover, thanks to fast fluidic exchange within the microfluidic system,²⁴ the diffusion of FISH probe from the probe solution to the targets situated in the tissue is also enhanced, resulting in short 4-h hybridization time (instead of an overnight incubation as in a standard FISH technique). The assay duration can be shortened to one working day (8 h) with the microfluidic method. Last but not least, it was shown that MA-FISH can be applied for either large centromere-targeted CEP17 probe or small locus-specific probe such as HER2 probe. Therefore, other FISH tests using small size range probes such as break apart-probes or single gene gain/loss probes could also be improved using the principle of MA-FISH.³⁶ Beside cost and duration, two other limitations of FISH method are the complexity of the procedure and the difficulty of interpretation. The latter is mainly based on direct microscopic counting by the user.^{37,38} This process is long, number of cell counted is limited and the counted cells are not traceable, ie, lost after the scoring is done. Developing automatic protocols, from the experimental staining procedure to signal scoring, would overcome these issues and improve the user-friendliness and reproducibility of FISH. Deconvolutions of z-stack images (*vide supra*) could pave the way for an automatic processing of FISH data. Overall, the intrinsic advantages of FISH such as technical robustness, and accurate interpretation are still retained for MA-FISH,¹⁹ and the microfluidic implementation of the technique lays the groundwork for the automation and dissemination of this technology.

In conclusion, the MA-FISH technique represents the first example of on-chip FISH analysis on tissue sections that decreases probe consumption and reduces processing times, offering significant improvements over the standard FISH method. The whole routine of *HER2* gene MA-FISH analysis on clinical breast cancer specimens could be performed in 1 day instead of the standard 2 days, thus improving the throughput and the screening capabilities. The system used here is still a prototype. Further technical implementations could focus at tissue microarrays, automation, or multiplexing to increase the MA-FISH throughput. The principle of using hydrodynamic flow for optimizing DNA hybridization provides the basis for improving further genetic investigations (chromogenic *in situ* hybridization, DNA microarrays, etc). Moreover, other diagnostic applications of FISH on cancer tissues, such as the analysis of *ALK* gene rearrangements in lung cancer, can also be improved using microfluidics, opening an alternative for facilitated analysis of the genetic state of the tumor. While precision medicine is opening a new era in cancer treatment, microfluidics provides the tools to decrease drastically the cost and time of genetic testing, thereby facilitating the dissemination of personalized therapy.

Supplementary Information accompanies the paper on the *Laboratory Investigation* website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

Professor Martin AM. Gijs has a patent application related to the technology described here (Swiss Patent Application 00256/12) and is involved in the start-up Lunaphore technologies SA developing this technology.

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