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ORIGINAL ARTICLE

Microfluidic-based immunohistochemistry for breast cancer diagnosis: a comparative clinical study

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Abstract

Breast cancer is a highly heterogeneous disease. The efficacy of tailored therapeutic strategies relies on the precise detection of diagnostic biomarkers by immunohistochemistry (IHC). Therefore, considering the increasing incidence of breast cancer cases, a concomitantly time-efficient and accurate diagnosis is clinically highly relevant. Microfluidics is a promising innovative technology in the field of tissue diagnostic, enabling for rapid, reliable, and automated immunostaining. We previously reported the microfluidic-based HER2 (human epidermal growth factor receptor 2) detection in breast carcinomas to greatly correlate with the *HER2* gene amplification level. Here, we aimed to develop a panel of microfluidic-based IHC protocols for prognostic and therapeutic markers routinely assessed for breast cancer diagnosis, namely HER2, estrogen/progesterone receptor (ER/PR), and Ki67 proliferation factor. The microfluidic IHC protocol for each marker was optimized to reach high staining quality comparable to the standard procedure, while concomitantly shortening the staining time to 16 min—excluding deparaffinization and antigen retrieval step—with a turnaround time reduction up to 7 folds. Comparison of the diagnostic score on 50 formaldehyde-fixed paraffin-embedded breast tumor resections by microfluidic versus standard staining showed high concordance (overall agreement: HER2 94%, ER 95.9%, PR 93.6%, Ki67 93.7%) and strong correlation (ρ coefficient: ER 0.89, PR 0.88, Ki67 0.87; $p < 0.0001$) for all the analyzed markers. Importantly, *HER2* genetic reflex test for all discordant cases confirmed the scores obtained by the microfluidic technique. Overall, the microfluidic-based IHC represents a clinically validated equivalent approach to the standard chromogenic staining for rapid, accurate, and automated breast cancer diagnosis.

Keywords Microfluidic tissue processor · Immunohistochemistry · Breast cancer

Introduction

Breast cancer is the most commonly diagnosed tumor and the leading cause of cancer death in women worldwide [1], accounting for almost one quarter of female cancer cases [2].

In combination with histological evaluation of standard hematoxylin-eosin staining, immunohistochemistry (IHC) of formaldehyde-fixed paraffin-embedded (FFPE) tissue biopsies has become an essential tool in oncologic pathology to guide personalized clinical management of patients. To analyze any putative breast cancer tissue, the expression of prognostic and therapeutic markers is routinely assessed, including the human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), progesterone receptor (PR), and proliferation factor Ki67 [3–5]. The efficacy of the therapeutic strategies relies on the precise detection and quantification of these diagnostic markers. Therefore, considering the increasing incidence of breast cancer cases, a concomitantly time-efficient

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and accurate assessment of biomarkers on tumor samples is of high clinical relevance.

Recently, we developed a microfluidic tissue processor (MTP) device and used to perform IHC and immunofluorescence (IF) staining on FFPE and frozen histological samples [6–8]. The microfluidic-based device is a pressure-driven system that allows reagents to be delivered on a biological sample via the fast-fluidic exchange (FFEX) technology, enabling for a precisely controlled immune-reaction to take place in an extremely short incubation time. In fact, the microfluidic IHC staining on frozen samples lasts less than 12 min [8]. Furthermore, the employment of the microfluidic-based device resulted in a better repartition of HER2 positive/negative breast carcinoma cases, thus reducing the number of equivocal HER2 scores by 90% and allowing for *HER2* gene copy number prediction [6, 7].

Here, we aimed to develop and validate a panel of microfluidic IHC protocols for the breast cancer diagnostic markers HER2, ER, PR, and Ki67. To this purpose, we performed a comparative clinical study using 50 successive eligible FFPE breast cancer resections to assess whether (i) the clinical diagnostic score and (ii) analytical performance achieved by the microfluidic staining device were equivalent to a routinely used BenchMark ULTRA automated stainer.

Material and methods

Breast cancer tissues

A total of 50 FFPE breast tumor resections retrieved from the files of Pathology Institute of the University Hospital of Lausanne (CHUV) were included in this study (ethical committee approval protocol number 511-12). Successive eligible cases were collected from January 2016 without any preselection criteria for biomarkers' grading. Tissue specimens were anonymized before being stained and blindly scored by a pathologist. The standard IHC and FISH assay were repeated for the purpose of the study, rather than reanalyzing the slides prepared during the diagnostic procedure.

Tissue slide pre-processing for MTP-based IHC

Tissue sections were dried for 10 min at 65 °C, dewaxed for 10 min 30 s in Histoclear (National Diagnostic, HS200), and rehydrated by consecutive washes in ethanol: 100% for 30 s, 100%, 95%, 70%, and 40% *v*/*v* for 10 s and then placed in MilliQ water. Heat-induced antigen retrieval was performed at 95 °C for 40 min at pH 6 (Dako, pH 6, S1699) for CK and Ki67 staining, and at pH 9 (Dako, pH 9, S2367) for ER, HER2, and PR staining. After cooling down to room temperature for 20 min, the samples were transferred in TBS before being placed on the microfluidic device.

Microfluidic-based IHC staining

The microfluidic device prototype used in this study was designed and manufactured by Lunaphore Technologies SA. The principle of operation of the microfluidic device employed for the IHC stainings is described in Fig. 1a. Optimized incubation time for all protocol step and primary antibody dilutions for the MTP-device are reported in Fig. 1b. MTP-based stainings were performed using custom-made HRP-conjugated secondary antibodies and DAB chromogenic substrates. Counterstaining was performed with 20% hematoxylin solution (Thermo Scientific, Gill3, 72604) in MilliQ water. All protocol steps were performed at 37 °C.

Primary antibodies

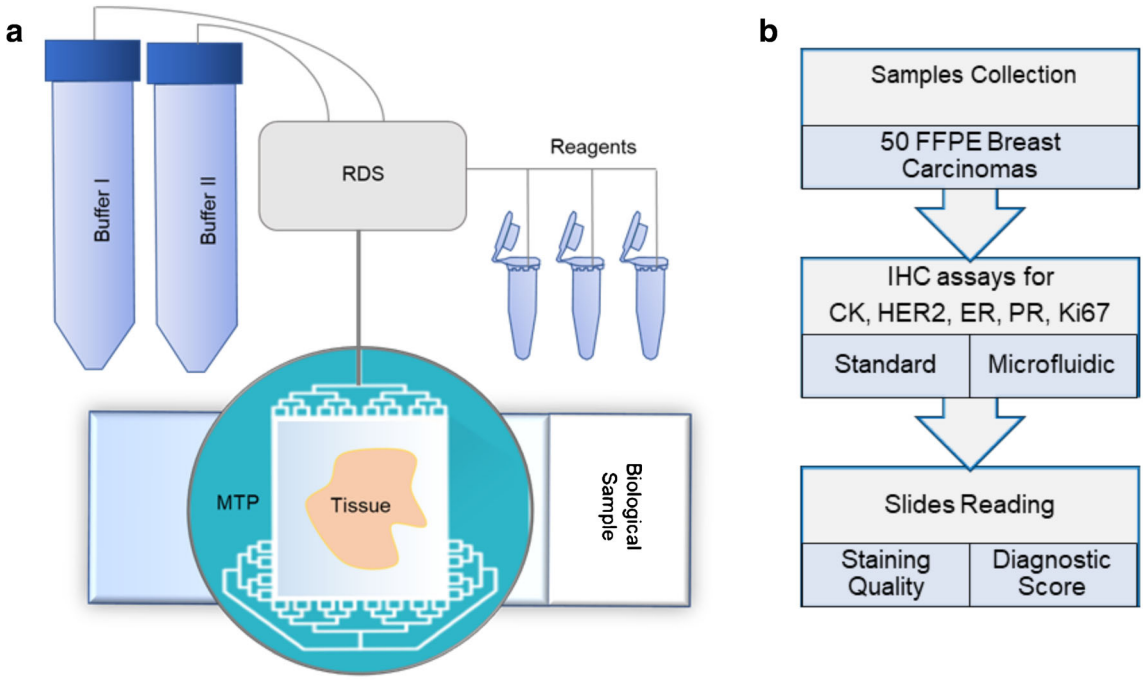
The primary antibody used in the study are as follows: mouse anti-human cytokeratin (CK) clone AE1/AE3 (Dako M3515, 1.07 mg/L), mouse anti-human ER clone 6F11 (Novocastra NCL-L-ER-6F11, 1.50 mg/L), rabbit anti-human HER2 clone 4B5 (Ventana 709-2991, ready to use), mouse anti-human Ki67 clone MIB-1 (Dako M7240, 0.92 mg/L), and mouse anti-human PR clone PgR-636 (Dako M3569, 0.81 mg/L). Primary antibodies were diluted in TBS 0.1% Tween (Fisher Bio-Reagents, BP337); working dilutions are reported in Fig. 1b. Same primary antibody batches were used both for standard and MTP-based stainings. The alternative primary antibody clones used for ER and PR standard staining on discordant cases were rabbit anti-human ER clone SP1 (Ventana 790-4325, ready to use) and PR clone 1E2 (Ventana 790-4296, ready to use).

Standard IHC pre-processing and staining

Standard IHC assays were performed on a BenchMark ULTRA automated stainer (Roche Ventana) according to manufacturer recommendations. Detailed protocols are provided in Suppl. Material and Methods.

Tissue slide IHC post-processing and image acquisition

The stained slides were rinsed with MilliQ water and dehydrated in ethanol 95%—ethanol absolute (Reactolab SA, 99570)—xylol (VWR Chemicals, 28973.363) baths (3 × 5 s each step). The slides were then mounted with Glas Mounting Medium (Sakura, Tissue-Tek, 1408) and coverslip 24 × 50 mm (VWR, ECN 631-1574) by using Tissue-Tek Glas g2-E2 (Sakura Finetek, 6502). The images were acquired with the digital slide scanner NanoZoomer S60 (Hamamatsu, C13210-01) and visualized with the Leica Biosystems Version 4.0.7 (Leica Biosystems).



c

Marker (Clone)		CK (AE1/AE3)		HER2 (4B5)		ER (6F11)		PR (PgR-636)		Ki67 (MIB-1)	
Staining Device		STD	MTP	STD	MTP	STD	MTP	STD	MTP	STD	MTP
Abl Dilution		1:100	1:67	RTU	RTU	1:50	1:50	1:25	1:100	1:50	1:50
Protocol Steps	H_2O_2	4		4		4		4		4	
	AbI	32	4	16	4	32	4	32	4	32	4
	AbII	8	4	8	4	8	4	8	4	8	4
	DAB	8	2	8	2	8	2	8	2	8	2
	$CuSO_4$	4		4		4		4		4	
	HTX	8	0.25	8	0.25	12	0.25	8	0.25	4	0.25
Total Time (min)		**132**	**16**	**116**	**16**	**144**	**16**	**144**	**16**	**132**	**16**

Fig. 1 Schematic representation of the microfluidic tissue processor (MTP) device, optimized protocols, and clinical study design. **a** The MTP device is based on an automated pressurized system allowing for rapid and uniform sequential delivery of reagents in a reaction chamber. The chamber of reaction (100 μm in height) is created at the interface between a microfluidic chip (MTP) and the glass slide carrying the tissue sample. This open-system allows the allocation of any suitable reagents either in four different 50 ml reservoirs (e.g., buffers) or in eight 1.5–2 ml reservoirs (e.g., antibodies). Reagents are dispensed and washed out by a pressure-driven flow via the microtubing system which is connected to the reagent delivery system (RDS) component. Reagents flow through the MTP system to reach the reaction chamber where the immunological reaction takes place. The exchange of reagents is done in a timeframe of 1 s due to the fast-fluidic exchange (FFEX) technology, which allows the immune-reaction to occur in an extremely short incubation time. After the incubation, the reagents were collected in the waste tube. **b** Microfluidic IHC staining protocols for CK, HER2, ER, PR, and Ki67 on FFPE breast cancer resections. Optimized incubation time for all protocol step and primary antibody dilutions are shown as compared to routinely used standard (STD) protocols. H_2O_2, oxygen peroxidase; AbI/II, primary/secondary antibody; DAB, 3, 3′-diaminobenzidine; $CuSO_4$, copper sulfate; HTX, hematoxylin. **c** Clinical study workflow

IHC quality parameters, diagnostic scoring, and exclusion criteria

The quality of IHC staining was evaluated on each slide, as perceived by an experienced pathologist, based on the following parameters: intensity, background, specificity, contrast, sharpness, morphology, and counterstaining. Intensity and sharpness were not evaluated in negative-scored samples. For each parameter, a mark from 1 (insufficient) to 5 (excellent) was assigned.

CK was scored in a binary way as positive or negative. The diagnostic scores for ER/PR, HER2, and Ki67 were evaluated according to 2010 ASCO/CAP guideline [9], 2013 ASCO/CAP guideline [10], and 2015 St. Gallen Conference recommendations [11], respectively. Detailed IHC quality and scoring parameters are provided in Suppl. Material and Methods.

Negative cases without internal positive control were considered technically unsatisfactory and discarded from the analysis (ER $n = 1$, Ki67 $n = 1$). Tissue sections that were ruined during the pre- or post-staining procedure resulting in unreadable stained cases were also excluded from the scoring comparison (CK $n = 1$, PR $n = 3$, Ki67 $n = 1$). All excluded cases are shown as strikethrough boxes in Suppl. Table 1.

Discordant scores

The diagnostic score was considered to be discordant when being inversely classified by the two employed staining techniques as positive/negative for ER and PR (cutoff 1% positive tumor nuclei), or high/low for Ki67 (cutoff 15% positive tumor nuclei). HER2 status was considered discordant when differently classified as negative, equivocal, or positive according to the scoring criteria defined above. HER2 discordant cases ($n = 3$) were further analyzed by FISH reflex test, according to the 2013 ASCO/CAP guideline recommendations [10]. Discordant cases for ER ($n = 2$), PR ($n = 5$), and Ki67 ($n = 8$) were blindly reviewed by a pathologist. For ER and PR, the cases remained discordant after rescoring were stained with an alternative primary antibody clone by the standard automated stainer; however, the resulting score was not used to redefine the overall agreement or correlation coefficient. Discordant diagnostic score values are shown in a bold case in Suppl. Table 1.

Dual-probe *HER2*/CEP17 FISH

FISH assay was performed as previously reported [12] using the *HER2*/CEP17 dual-labeled DNA probe (PathVysion *HER2* DNA Probe Kit, Abbott Molecular). Images were acquired using the Zeiss fluorescent microscope at high resolution (×63 objective, 1.4 numerical aperture). Results were interpreted according to the 2013 ASCO/CAP recommendations [10].

Results

Optimization of the IHC protocol for microfluidic-based staining of breast cancer diagnostic markers

The microfluidic device is an automated pressure-driven system allowing for fast and uniform delivery of reagents on the tissue section, thus resulting in a precisely controlled immunereaction within an extremely short incubation time enabled by the fast-fluidic exchange technology (Fig. 1a).

Microfluidic IHC for pan-cytokeratin (CK), commonly used to identify tumor epithelial cells, was firstly developed as a positive control marker on FFPE breast carcinoma cases (Suppl. Fig. S1). Microfluidic staining protocol for the diagnostic markers HER2, ER, PR, and Ki67 was subsequently optimized to reach a detection level comparable to the reference staining obtained from a routinely used standard automated stainer (Suppl. Fig. S2). In parallel, a negative control was performed by using the same staining protocols without adding the primary antibody. The obtained stainings displayed the absence of chromogenic signal and adequate counterstaining (Suppl. Fig. S2 and S3). The optimized staining conditions for all markers assessed in this study are reported in Fig. 1b, in comparison to routinely used standard protocols. Staining time for the optimized microfluidic protocol was shortened to 16 min, resulting in 5 to 7 folds decrease in time with respect to the BenchMark ULTRA protocols (Fig. 1b)—not including the tissue section deparaffinization and antigen retrieval pre-processing steps.

Reproducibility of the microfluidic-based IHC

To assess the reproducibility of the IHC protocol performed on the microfluidic device, a day-to-day repeatability test was run on three different days (with unchanged operator and location) for (i) CK using three different breast carcinomas—in parallel with negative controls without primary antibody (Suppl. Fig. S4a), and (ii) HER2, PR, and Ki67 on two different tissues (Suppl. Fig. S4b). The multiple IHC assays performed on different days resulted in a comparable pattern of expression and quality of staining on all tissue replicates for the assessed markers. Furthermore, to evaluate the consistency of the microfluidic staining for diagnostic purposes, the HER2, PR, and Ki67 stained tissues were clinically scored. HER2 score for both samples (0 and 3+) was identical on the three replicates; one sample triplicates showed equal score for PR (80%) and Ki67 (10–15%), while the other sample varying score (PR 100-95-95%, Ki67 40-35-30%), with a standard deviation of 3 (coefficient of variation, CV 0.03) and 5% (CV 0.14), respectively.

Analytical and diagnostic performance comparison

To assess the clinical reliability of the stainings performed by the microfluidic device, 50 FFPE surgically resected invasive breast carcinoma specimens were stained for CK, HER2, ER, PR, and Ki67 and subsequently scored for both analytical and diagnostic performance, in comparison to a standard automated stainer routinely used in tumor diagnostics (Fig. 1c). The optimized protocols, as defined in the above paragraph, were employed for the microfluidic-based assays. The brown tone of the microfluidic staining shifted towards a reddish brown, whereas the standard staining towards brown-black, possibly due to the usage of the copper sulfate signal enhancer on the BenchMark-stained samples. Yet, the developed microfluidic

protocol achieved high qualitative performance equivalent to the routine assay for all assessed markers (Suppl. Fig. S5).

As expected for breast carcinomas, all stained samples with both the MTP and BenchMark devices were cytokeratin positive (Suppl. Table 1).

HER2 Employment of the microfluidic device resulted in a high-quality HER2 staining comparable to the standard assay (Fig. 2a, b) for all considered parameters as shown in Suppl. Fig. S5. Staining results for HER2 IHC were evaluated according to the 2013 ASCO/CAP guideline recommendations, with HER2 status being classified as positive (3+ score), equivocal (2+ score), or negative (1+ and 0 score). Comparison of HER2 diagnostic score for microfluidic-based versus standard staining assay showed an overall agreement of 94%, with 47 cases (35 negative, 9 equivocal, and 3 positive) out of 50 concordantly classified by the two techniques (Fig. 2c and Suppl. S6a). One of the three discordant cases was classified negative (1+) by the standard staining and equivocal (2+) by the microfluidic staining; vice versa, the other two discordant cases were classified equivocal (2+) by the routine assay and negative (1+) by the MTP-based assay (Fig. 2d and Suppl. Table 1). FISH analysis confirmed the *HER2* status to be concordant with the scoring assessed by the microfluidic staining assay for all three analyzed cases (Fig. 2d and Suppl. Table 1). As recommended by the ASCO/CAP guidelines, all eight cases concordantly scored as equivocal by both IHC techniques were also analyzed via FISH assay (one case was not assessed due to lack of biological material). Six cases were confirmed to be *HER2* equivocal by FISH analysis, one was *HER2* amplified, and one was *HER2* negative (Suppl. Fig. S6b).

ER The microfluidic-based assay showed a high qualitative ER staining performance comparable to the standard staining (Fig. 3a, b) for all considered parameters (Suppl. Fig. S5). Evaluation of the ER-positive/negative status (cutoff, 1% positive tumor nuclei) for the microfluidic-based versus standard staining techniques showed an overall agreement of 95.9%, with 47 out of 49 concordantly scored cases (Fig. 3c and Suppl. Table 1). Two out of 49 specimens were differently classified for ER status using the two staining techniques: ER-negative (both 0%) by standard staining and ER-positive (2 and 20%) by MTP staining (Fig. 3c). The microfluidic-based staining detected a higher percentage of ER-positive nuclei in samples with low to moderate positivity by standard assay. Nevertheless, pairwise comparison of the assessed percentage of positive tumor nuclei employing the two staining techniques resulted in a strong positive correlation with a Pearson coefficient (ρ) equal to 0.89 (p value < 0.0001).

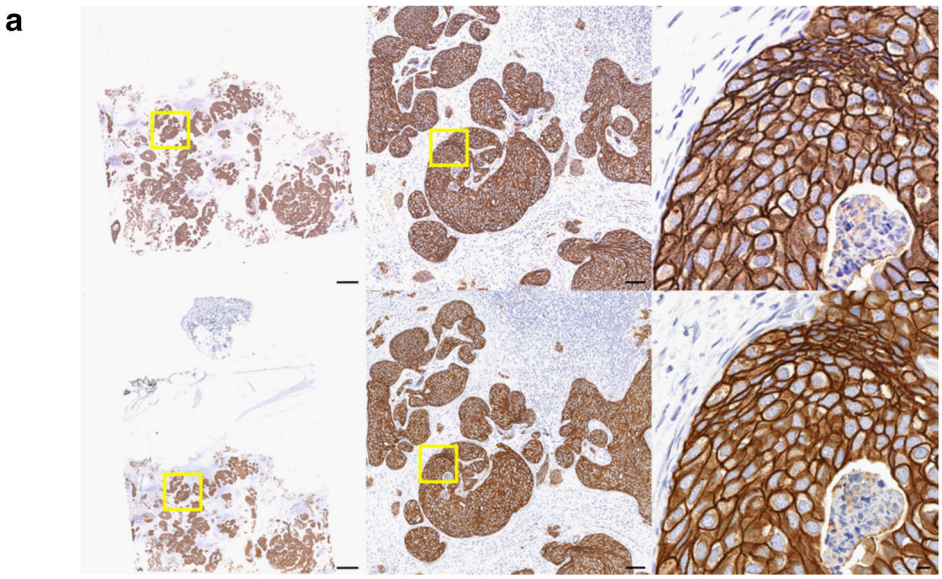
PR The MTP-based assay showed a high qualitative PR staining performance comparable to routine methodology as shown in Fig. 4a, b and Suppl. Fig. S5. Evaluation of the PR-positive/negative status (cutoff, 1% positive tumor nuclei) as assessed by the microfluidic versus standard assay showed an overall agreement of 93.6%, with three out of 47 cases being discordantly classified (Fig. 4c and Suppl. Table 1). The three discordant cases were classified as PR-negative (all scored 0%) after standard IHC while PR-positive (one case 3% and two cases 5%) after microfluidic-based assay (Fig. 4c). The MTP-based PR staining, although using four times less concentrated (1:100) primary antibody than the BenchMark (1:25), resulted in a higher percentage of PR positive nuclei in samples displaying low to moderate positivity by standard assay. Nonetheless, a pairwise comparison of the scored percentage of positive tumor nuclei resulting from two assays showed a strong positive correlation with a Pearson coefficient (ρ) equal to 0.88 (p value < 0.0001).

Ki67 The MTP-based immunoreaction resulted in a high qualitative Ki67 staining performance (Fig. 5a) equivalent to the standard assay (Suppl. Fig. S5). The Ki67 proliferative index was computed as the percentage of positive tumor cell nuclei (Fig. 5b), as it is regularly evaluated as a continuum value for clinical decisions contingently to its intended use. Pairwise comparison of the percentage of Ki67-stained tumor nuclei as assessed by two IHC techniques resulted in a strong positive correlation with a Pearson coefficient (ρ) equal to 0.87 (p value < 0.0001). For Ki67 score dichotomization as high/low, it was defined a data-derived cutoff point of 15% Ki67-positive nuclei, corresponding to the sample population median value as per standard assay; an equal median value resulted from the microfluidic-stained population. Three cases out of 48 were differentially classified as Ki67 high/low with an overall agreement of 93.7%. All three discordant cases were classified as Ki67-low (score 10%) by standard assay, while Ki67-high (one case 20% and two 15%) by microfluidic-based IHC (Suppl. Table 1).

Discussion

In this study, we proved the clinical validity of the microfluidic-based IHC assay as an equivalent approach to the standard chromogenic staining, allowing for breast cancer diagnosis by automated, rapid, and accurate detection of HER2, ER, PR, and Ki67.

Immunohistochemistry is a fundamental methodology in the diagnostic process of solid tumors. The need for IHC is constantly intensifying due to increasing cancer incidence and a higher number of biomarker assessments per patient, according to recent trends in personalized medicine. The instruments commonly used for IHC on clinical specimen require hours to perform the staining process. Employment of the microfluidic tissue processor device allowed to perform IHC staining of



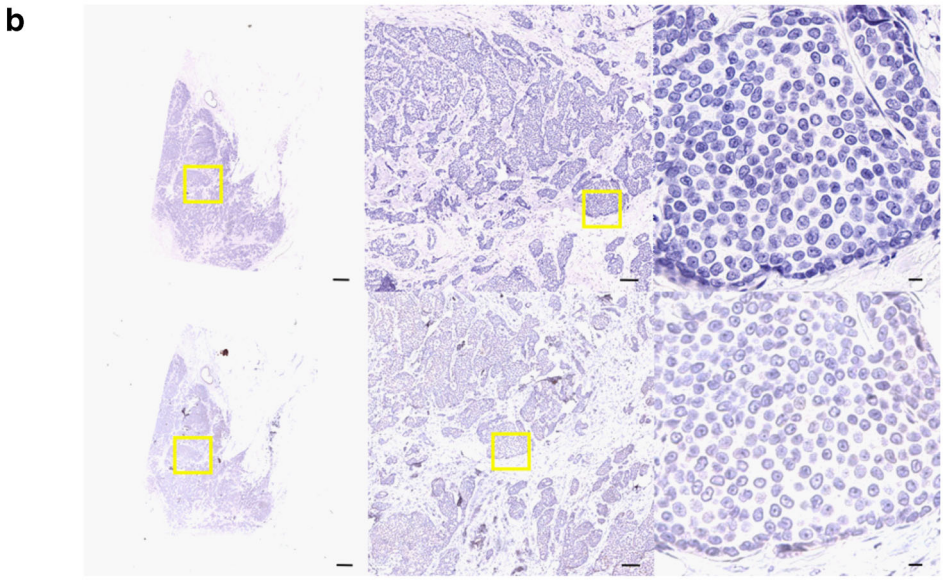


Fig. 2 HER2 staining comparison (**a**, **b**). Shown is a representative breast carcinoma (**a**) HER2 positive (score 3+) and (**b**) HER2 negative (score 0) by standard (upper panels) or MTP-based (bottom panels) IHC assay. Scale bar: 1 mm, 100 μm, and 10 μm (left to right). **c** HER2 IHC scoring table. Concordantly scored cases (highlighted in gray) by MTP and standard assay: 35 negative (0–1+), 9 equivocal (2+), and 3 positive (3+); discordantly scored cases are in blue. **d** HER2 scoring comparison: IHC versus FISH. HER2 scoring by standard (STD), microfluidic (MTP), and dual probe (*HER2/CEP17*) FISH assay for the three discordant cases in **c**; highlighted in gray are HER2 concordantly scored cases by MTP and FISH assay. The HER2 diagnostic score values for all cases arranged by chronological order (1–50) are reported in Suppl. Table 1

c

HER2 Scoring		Standard IHC		
		0-1+	2+	3+
MTP IHC	0-1+	**35**	2	0
	2+	1	**9**	0
	3+	0	0	**3**

d

HER2 Score for Discordant Cases		
STD IHC	MTP IHC	FISH
1+	2+	Equivocal
2+	1+	Negative
2+	1+	Negative

breast cancer biopsies in less than 16 min—apart from deparaffinization and antigen retrieval step—with a time reduction down to 7 folds. A turnaround time lower than the state-of-the-art techniques opens the possibility of obtaining higher throughputs in clinical laboratories, reducing the congestion in healthcare systems.

The configuration of the microfluidic device prototype, at the time of this study, required a reagent volume double as compared to the standard automated stainer, resulting in economic disadvantage other conditions being equal. However, this was in part counterbalanced by the lower concentration of the primary antibody used in the microfluidic device

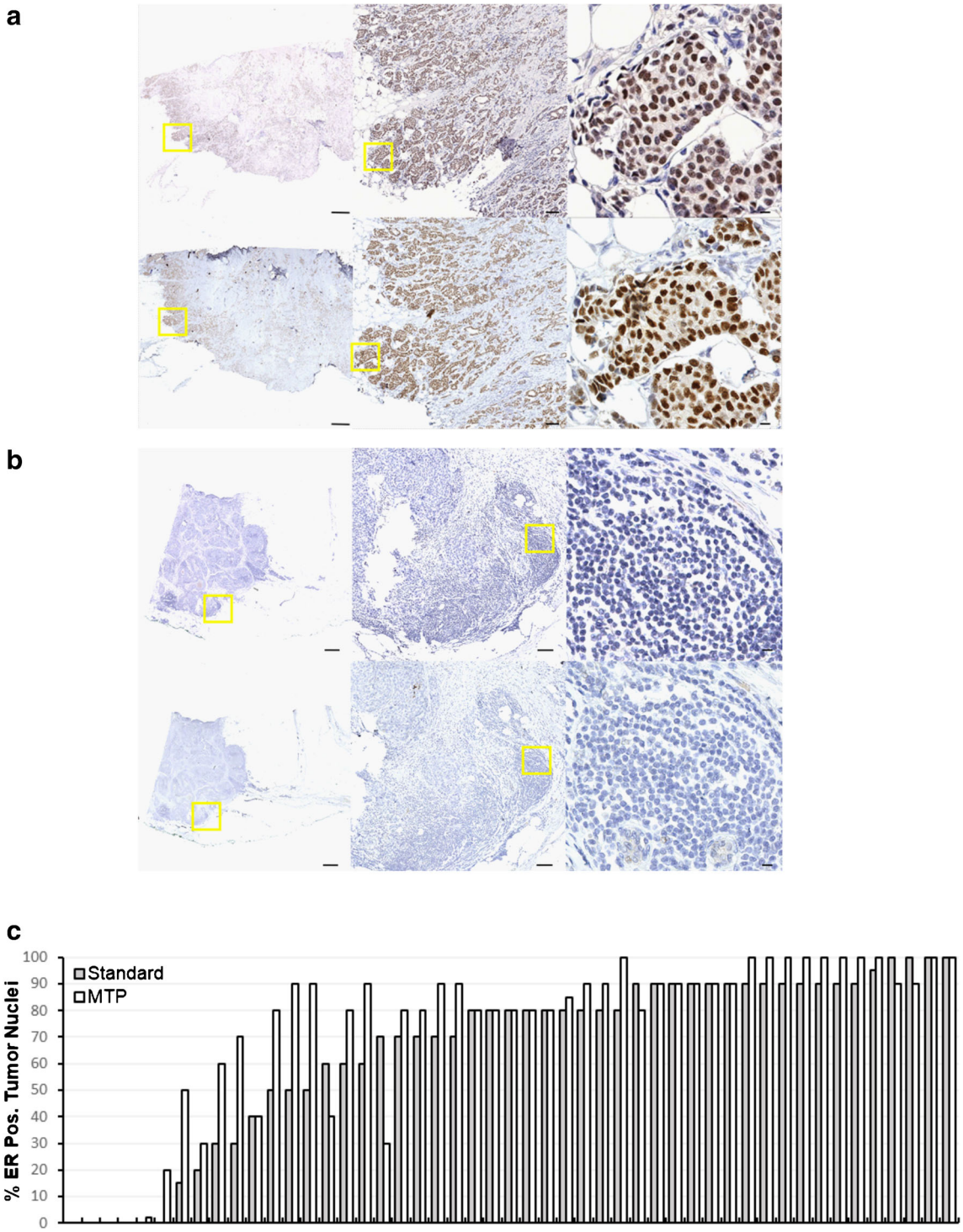


Fig. 3 ER staining comparison (**a**, **b**). A representative breast carcinoma **a** ER-positive (90% positive nuclei) and **b** ER-negative by standard (upper panels) or MTP-based (bottom panels) IHC assay. Scale bar: 1 mm, 100 μm, and 10 μm (left to right). **c** ER scoring comparison. The percentage of ER-positive tumor cell nuclei as assessed by a standard or microfluidic (MTP) assay on evaluable cases ($n = 49$); cases are arranged by increasing ER scoring as per standard assay. The ER diagnostic score

protocols, e.g., down to four times less for PR. Moreover, the next generation of the microfluidic device includes improvements for reducing the reagent consumption to be leastwise comparable to the standard techniques.

The HER2 microfluidic staining in breast carcinoma was previously reported to better correlate with the *HER2* gene amplification status as assessed by FISH [6, 7]. Consistently with prior observations, all discordant cases were confirmed to

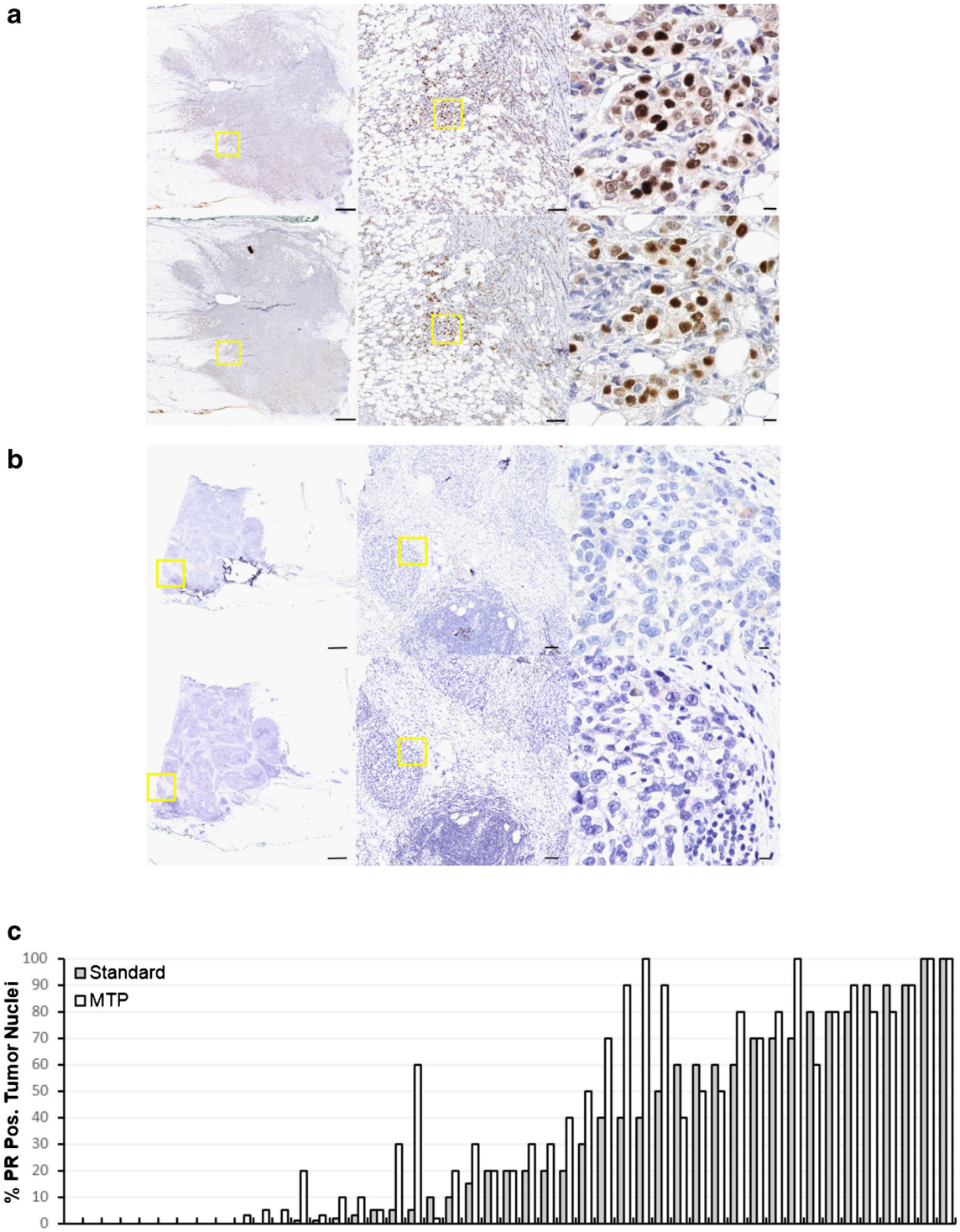


Fig. 4 PR staining comparison. **a** A representative breast carcinoma positive for PR by standard (upper panels, 60% positive nuclei) or MTP-based (bottom panels, 40% positive nuclei) IHC assay. Scale bar: 1 mm, 100 μm, and 10 μm (left to right). **b** A representative breast carcinoma negative for PR by standard (upper panels) or MTP-based (bottom panels) IHC assay. Scale bar: 1 mm, 100 μm, and 10 μm (left to right). **c** PR scoring comparison. The percentage of PR-positive tumor cell nuclei as assessed by standard or microfluidic (MTP) assay on evaluable cases ($n = 47$); cases are arranged by increasing PR scoring as per standard assay. The PR diagnostic score values for all cases arranged by chronological order (1–50) are reported in Suppl. Table 1

be correctly classified by MTP staining when re-analyzed by FISH assay, both for lower (1+ versus 2+) and higher (2+ versus 1+) HER2 scored samples.

Being the primary aim of the study to prove the equivalency of the microfluidic IHC to the standard assay in a real clinical setting, the specimens were collected from a hospital

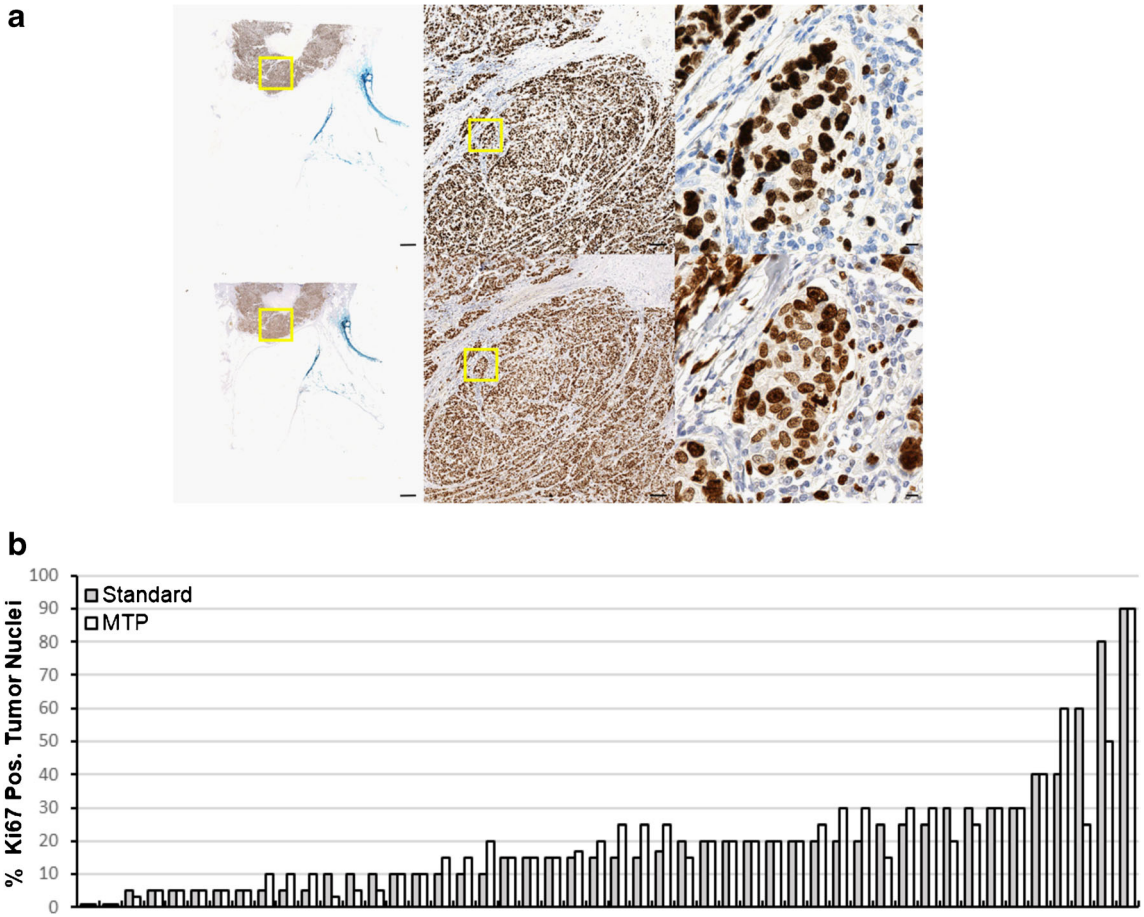


Fig. 5 Ki67 staining comparison. **a** A representative breast carcinoma stained for Ki67 (90% positive nuclei) by standard (upper panels) or MTP-based (bottom panels) IHC assay. Scale bar: 1 mm, 100 μm, and 10 μm (left to right). **b** Ki67 scoring comparison. The percentage of Ki67-positive tumor cell nuclei as assessed by standard or microfluidic (MTP) assay on FFPE breast carcinoma ($n = 48$); cases are arranged by increasing Ki67 scoring by standard assay. The Ki67 diagnostic score values for all cases arranged by chronological order (1–50) are reported in Suppl. Table 1

diagnostic facility as chronologically accessible. Of note, the distribution of the cases across the scoring categories was unbalanced with a small proportion of HER2-positive carcinomas (i.e., 3/50 cases), providing limited statistical power in consideration of individual categories; however, this was inherent to the design of the study and reflected the biomarker distribution in the actual population.

Stratification of HER2-negative results by the assigned score, 0 or 1+, showed about half of the cases (15/35) differentially scored by the two IHC assays, with most of the cases (13/15) scored as 1+ by the MTP while 0 by standard HER2 staining. In a similar manner, the MTP staining could detect a higher number of ER and PR positive nuclei in samples showing low to moderate percentage of positivity by standard assay, being for PR even more remarkable due to four times lower primary antibody concentration in the MTP staining. These data suggested for the microfluidic device to enable for higher sensitivity as compared to the standard assay. This feature could partially account for the ER and PR discordantly scored samples, which were in all cases classified as negative by BenchMark while positive by MTP device. Among others, sensitivity and specificity of different primary antibodies clones are important parameters for IHC accuracy [9, 13]. The anti-human ER rabbit monoclonal SP1 antibody was previously reported to exert a greater sensitivity compared with other clones, including 6F11 [13]. Standard IHC assay of the ER discordant cases with the SP1 clone confirmed one case to be positive as assessed by MTP, while the other one remained negative (data not shown). In previous studies, the anti-human PR rabbit monoclonal 1E2 antibody showed a higher analytical affinity for PR with respect to the 636 clones [13, 14]. Standard staining of PR discordant cases with the 1E2 clone confirmed two of the three cases to be positive as assessed by microfluidic assay, while one remained negative as by standard staining (data not shown). Remarkably, the two cases reassessed with the 1E2 clone as positive showed the same percentage of positive nuclei as for the MTP-based assay (5% for both samples). Keeping the

detection system fixed for the standard assay when performing the staining with a different primary antibody clone ruled out the possibility that apparently higher sensitivity was due to the employment of different detection system for the two assays. The ER/PR positivity after standard staining with alternative clones suggested for higher sensitivity of the MTP that could be counterbalanced by supposedly more sensitive primary antibodies for the standard assay. Unlike for HER2, there is no accepted alternative assay for assessing ER and PR status for discordant cases [13]; therefore, the score resulting from alternative clones was not used to redefine the agreement or concordance. The higher sensitivity of the microfluidic device could represent a high added value with potential economic and health benefits, e.g., the false negative proportion for hormone receptor ER/PR status in breast cancer was reported to be 20–30% [15]. Testing of such a possibility will require further investigation on a validation set enriched in low-expression and negative samples to be correlated with clinical outcome. The three Ki67 discordant cases were classified as high by the MTP while low by standard assay; however, in this case, there was not a trend for higher Ki67 percentage detected by the microfluidic device in the analyzed sample population.

Semi-quantitative measurement of HER2, ER/PR, and Ki67 is relevant for IHC-based surrogate subtype classification of breast cancer in HER2-positive, luminal A-/B-like, or triple negative for prognosis and therapeutic strategy determination. According to the latest criteria proposed by Prat et al. [16] and accepted in the 13th St Gallen International Breast Cancer Conference [17], one case would be discordantly subtyped as luminal B versus A and two cases as luminal A versus B according to PR (cutoff 20%) and Ki67 (cutoff 14%) status as determined by standard or microfluidic assay, respectively.

Overall, here we proved the rapidity and non-inferiority of the microfluidic IHC with respect to standard assay for breast cancer diagnosis. Furthermore, being the microfluidic device an open-system, it represents an effective tool for validation and clinical use of any current and emerging biomarkers for tissue diagnostics.

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Authors' contributions S.B., D.G.D., M.G., N.P., and L.d.L designed the study. S.B. and N.P. supervised study execution. F.A. ran the experiments. M.T.A.F. and J.P.B. blindly scored the assessed cases. M.G.P. interpreted the results, F.A. and M.G.P designed the figures, and M.G.P and F.A. wrote the paper. All authors contributed to manuscript writing and revision, and they all approved the submitted version. The authors declare that the article is presently not under consideration for publication in another journal and has not been published previously.

Compliance with ethical standards

Conflict of interest At the submission of the paper, DGD and SB are employed by Lunaphore Technologies SA, which is commercializing a microfluidic-based staining device. DGD and MAMG have equity interest in Lunaphore Technologies SA. The other authors declare no conflict of interest.

Ethical approval A total of 50 FFPE breast cancer tissue specimens were retrieved from the Pathology Institute of the University Hospital of Lausanne (CHUV) between January 2016 and April 2017 with the ethical committee approval (protocol number 511-12).

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