CHARACTERIZATION OF SHORT INCUBATION TIME EFFECTS ON CHROMOGEN SIGNAL OBTAINED BY HER2-EXPRESSING BREAST CARCINOMAS USING MICROFLUIDIC IMMUNOHISTOCHEMISTRY

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ABSTRACT

We present a microfluidic tissue processor (MTP) for detection of the human epidermal growth-factor receptor 2 (HER2) with immunohistochemistry (IHC) on clinical breast cancer tissue sections using chromogenic detection. In particular, we investigate the effect of short incubation times in resultant chromogenic signal for an accurate quantification.

KEYWORDS

Immunohistochemisrty, microfluidics, chromogenic detection, human epidermal growth-factor receptor 2 (HER2/*neu*)

INTRODUCTION

HER2 is a kinase bound to the cell membrane, whose overexpression in breast carcinoma tissue connotes diagnostic and prognostic information [1]. Therefore, quantitative analysis based on the intensity of the HER2-IHC signal is routinely done in breast carcinomas [2]. Recently, we have shown that, when short antibody incubation times (t_{inc}) are used during subsequent immunoassay steps of an IHC protocol applied by our MTP, shown in Figure 1. HER2 quantification can be much more accurate [3]. Nevertheless, in this system, fluorescently conjugated secondary antibodies were used for detection, while in current clinical practice, horseradish peroxidase (HRP)-labeled secondary antibodies with subsequent chromogen precipitation is employed.

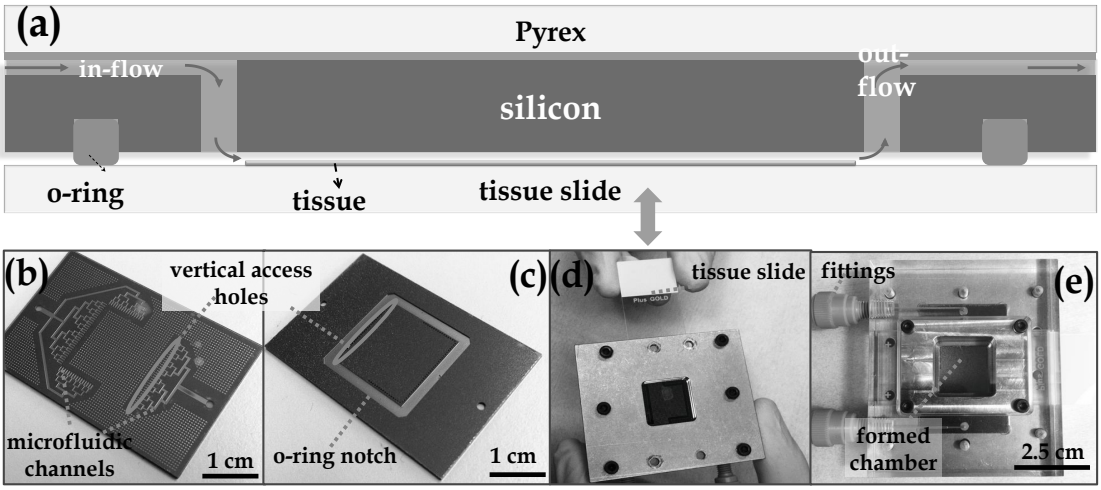

Figure 1: Microfluidic tissue processor (MTP) (a) Illustration of the microfluidic chip cross-section, fluidic inlet and access holes sealing o-ring, and their integration with a tissue section. (b) Top view of the chip showing the microfluidic distribution channels; (c) inverse view of the chip that shows the o-ring zone and access hole connections to the chamber, against which the tissue slide is clamped. (d) Assembled system during insertion of the tissue slide, and (e) after clamping with the tissue slide, ready for processing. Figure partially adapted from [3], Copyright 2003 National Academy of Sciences, USA.

If the working principle behind the accuracy increase provided by the MTP can also be observed when using chromogenic detection, its integration to clinical practice would be much more straightforward in terms of user education, training of the scoring pathologist, use of existing pathology microscopes and comparing outcomes with previously obtained results. Previously demonstrated benefits of a short t_{inc} on the resultant signal accuracy were based on fine-tuning of immunoreaction kinetics only and on quantitation of the fluorescent signal [3]. In case of fluorescence, the signal intensity of an image can be taken proportional to the number of fluorophore-conjugated secondary antibodies. However, the contrast generated by a chromogenic precipitate, when observed in a bright-field microscope, possibly has a much more complex dependence on the concentration of the secondary antibody, and additionally on the chromogenic substrate concentration and incubation time t_{chro}.

EXPERIMENTAL

We first optimized the time t_{inc}, by using a HRP-labeled secondary antibody system in combination with

diaminobenzidine (DAB) substrate applying an on-chip protocol illustrated in Figure 2. Briefly, the protocol starts off-chip with dewaxing, and rehydration in successive 100%, 95%, 70%, 40% ethanol solutions, followed by a 10 min heat-induced epitope retrieval performed at 95 ˚C in a sodium-citrate buffer. After cooling down to room temperature during 20 min, the sections are transferred to the MTP, and the on-chip protocol starts with incubating primary antibody (DAKO polyclonal rabbit anti-human c-erB-2 oncoprotein, A0485). In the previously reported protocol, incubation is done with a fluorescent conjugated secondary antibody (Alexa 594-labeled goat anti-rabbit IgG antibody, A-11037), and the sections are transferred to the fluorescent microscope after coverslipping. On the other hand, the chromogenic protocol requires incubation of a HRP-conjugated secondary antibody, followed by exposure to diaminobenzidine (DAB) (DAKO Envision system). The HRP reacts with DAB and this reaction results in a colored product.

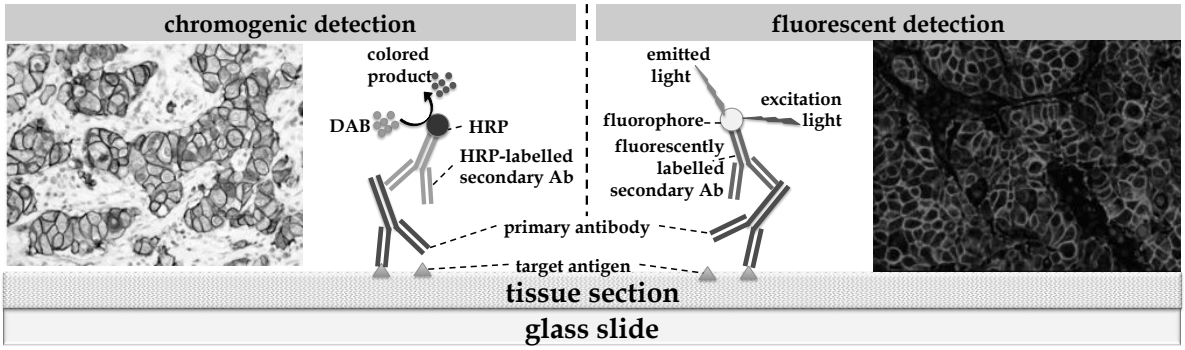

Figure 2: Comparison of on-chip HER2 immunohistochemistry protocols employing chromogenic and fluorescent detection. The schematic diagrams at the center illustrate the protocols used to observe HER2 positivity in the cell membranes using either chromogenic or fluorescent detection. The representative images on the left and the right hand side show typical HER2-positive tissue section after exposure to a chromogenic and fluorescent protocol, respectively. Images are 350 µm wide.

We have logarithmically swept $t_{inc} = 3 \times t_{chro} = 2^n$ min with n = {0,1,2,3,4,5} using tissue sections that were adjacently cut from a strong HER2-positive tissue. Hereafter, the images were acquired by using a bright-field microscope. We quantified the chromogenic signal, as illustrated in Figure 3**,** and plotted it versus t_{inc} as given in Figure 4b. Moreover, as the chromogenic incubation, in which the chromogen is precipitated during a time t_{chro}, is not an immunoreaction, the time scale of the reaction kinetics and diffusion constants may differ significantly from those of the antibodies. Hence, another study was done for finding the best t_{chro} independent of t_{inc}. In this case, we have fixed $t_{inc} = 4$ min, swept only $t_{chro} = 2^n/3$ min, and plotted the signal versus t_{chro} as shown in Figure 4c.

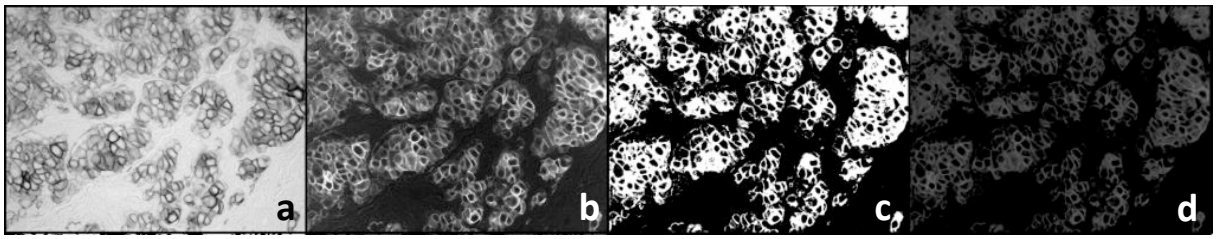

*Figure 3: Calculation of the IHC signal intensity of tissues subjected to a chromogenic detection protocol with the MTP. In order to calculate the signal intensity obtained from the chromogenic detection technique, we started by obtaining **(a)** a bright-field grey-scale image processed section, and **(b)** later inverted this image to obtain a dark-field image as in the fluorescent case. Since contrast of a bright-field image depends on many other factors, in addition to the presence of the colored product, we first identified HER2 expressing regions by thresholding the image shown in (b) (using the built-in threshold function of the open source software ImageJ), which results in a binary mask, as shown in **(c)**. **(d)** Finally, we obtained an image by multiplying the images shown in (b) and (c), and we took the average of the pixel values of this image as the signal intensity. Such calculated signal values from 100 images obtained from a tissue section are taken as the signal intensity of the tissue. Images are 400 µm wide.*

The obtained chromogenic signal trend after the application of the protocols in Figure 4b and 4c, is characterized by a linear and saturated part as a function of t_{inc} and t_{chro}, respectively. This is consistent with the results obtained from fluorescence experiments shown in Figure 4a. In general, an initial fast signal increase is followed by a slower one [4], where the immunoreaction rate is limited by the transport of the antibody to the tissue surface; this transport limitation is due to the formation of an antibody depletion close to tissue [5]. Therefore, we anticipate that the signal development rate in this transport-limited regime is not proportional to the amount of target antigen on the surface, but to the diffusion constant of the antibodies. Note that, while the signal trends are similar, the time scale differs in particular in Figure 4c, indicating both a faster substrate-chromogen diffusion and reaction with HRP, when compared with the immunoreactions.

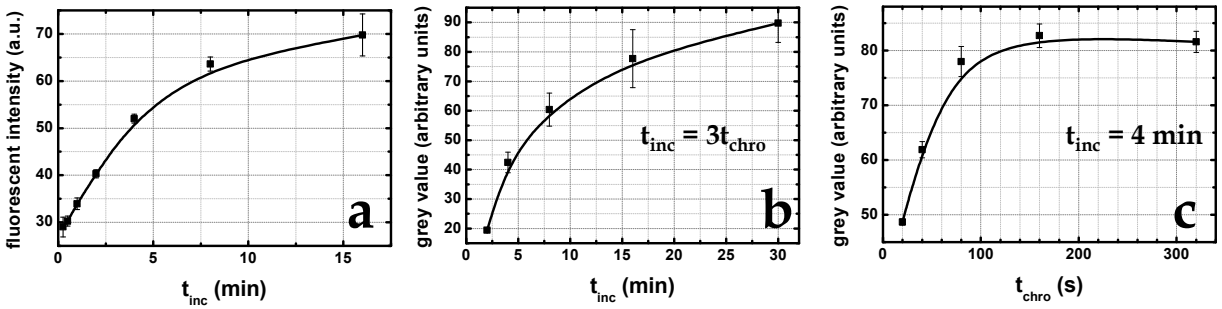

*Figure 4: IHC signal intensity as a function of t_{inc} and t_{chro}. (a) Fluorescent signal intensity versus t_{inc} as previously obtained in [3] (data points are for n=-2,-1,0,1,2,3,4). (b) Chromogenic signal intensity versus t_{inc} and (**c**) chromogenic signal intensity versus chromogen incubation time $t_{chro,}$ when t_{inc} is kept constant at 4 minutes (data points are for n=0,1,2,3,4,5). In these curves, data points are calculated by processing images as explained in Figure 3, and the error bars indicate standard deviations*

CONCLUSIONS

We investigated the effects of short incubation times in resultant chromogenic signal for an accurate quantification and compared it with those obtained with a fluorescent-signal study. Our results suggest that the proportionality that dominates the initial minutes of the reaction kinetics [5-6] can be exploited independent of used exact detection technology. Therefore, we can conclude that our MTP is also a promising tool that can be easily integrated with current clinical diagnosis protocols that involve chromogenic detection steps.

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