LabChip - Electronic Supplementary Information

Micro-immunohistochemistry using a microfluidic probe

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The vertical microfluidic probe platform.

The vMFP setup (see **Fig. S1**) used was an assembly of 4 main components: (1) A vertically mounted microfluidic chip called the vMFP head, (2) high precision syringe pumps for liquid handling (Cetoni GmbH, Korbussen, Germany), (3) a computer controlled X-Y-Z-stage built from motorized linear drives with 0.1 µm resolution (Lang GmbH, Huettenberg, Germany), and (4) an inverted fluorescence microscope (Nikon Eclipse TE300, Egg, Switzerland). The head was housed within a holder (see **Fig. S2**) mounted to the Z-drive via a tilt adjustment unit. The holder was equipped with ports enabling liquids to be pumped from or to the vMFP head via capillaries (1/32 inch outer dia, 0.005 inch inner dia). A custom-made frame, attached to the X- and Y-drives was used to mount standard microscope glass slides (1 × 3 inches) and for enabling the movement of the sample relative to the head in X and Y. The entire platform was controlled using a graphical user interface developed in LabView 2010.

The vMFP was operated using the following procedure: The fluid system (syringes, capillaries, vMFP head) was purged using water to eliminate air in the system. Then the vMFP head was leveled relative to the glass slide comprising a tissue section and the zero position established, this procedure is detailed elesewhere[13]. The vMFP head was elevated to ~50 µm above the glass slide and after pipetting a few ml of immersion liquid (buffer) onto the surface, the aspiration was started with a flow rate of 5 µl min^{-1}. After approximately 30 s, the injection with the antibody solution (1 µl min^{-1}) was initiated. A stable flow confinement was formed within less than a minute.

Design and fabrication of the vMFP head.

The vMFP head is a two layer (Si/glass) microfluidic device comprising microchannels (flow paths), vias for fluidic connection and a polished edge (apex) where the microchannels exit into open space (apertures). The apex physically supports the flow confinement, and has an area of approximately 1 mm^2. The microchannels are typically 50 µm deep and tapered from 200 µm width (starting from a via) to 50 µm (at the apertures). This design can easily be changed as needed and several variants of heads are described elsewhere[13].

Fabrication of vMFP heads was done using standard microfabrication techniques (photolithography, deep reactive ion etching and anodic bonding). The microchannels and vias were etched in a 500-µm-thick 4 inch Si wafer (Siltronix, Geneva, Switzerland). The

microstructured Si wafer, with 33 vMFP heads was then anodically bonded (450 ˚C, 1.25 kV) to a 500-µm-thick glass wafer (Borofloat® 33, SCHOTT AG, Germany). The microstructures were filled with 80 °C molten wax (OCON 199, Logitech GmbH, Germany) for protection during dicing and polishing of the heads. Heptane was used to remove the wax from the microstructures of the polished heads.

Tissue processing and conventional IHC procedure.

The paraffin embedded tissue sections were dewaxed by placing the slides for 3 min in xylene followed by 3 min in a second xylene bath. For rehydration, the tissue sections were then placed in xylene/ethanol (1:1 vol) for 3 min, 3 min in 95 % ethanol, 3 min in 70 % ethanol and 3 min in 50 % ethanol. The tissue sections were then rinsed with tap water for approximately 3 min. Antigen retrieval was then performed by immersing the slide in citrate buffer (10 mM citric acid, 0.5 % Tween 20, pH 6.0) and placing it into an autoclave at 95 °C for 2 min. The tissue sections were then washed and stored in PBS-Tween 20 (0.5 % v/v). A mouse specific HRP/DAB detection IHC kit (ab64259, Abcam, Cambridge, UK) was used as the biochemical detection system for further processing of the tissue (further details in **Fig. S3**). A protein block solution from the IHC kit was applied to the tissue sections for 10 min at room temperature followed by a quick rinsing step using PBS-Tween 20 (0.5 % v/v). The tissue sections were then incubated with the monoclonal primary antibody diluted with the antibody diluent for durations suggested for specific antibodies. After the incubation, the tissue sections were rinsed with PBS-Tween 20 (0.5 % v/v) 3 times for 2 min and then IHC processing for visualization with the HRP/DAB kit was performed. For this, the tissue sections were incubated with hydrogen peroxide block solution for 10 min and rinsed with PBS-Tween 20 (0.5 % v/v) followed by incubation with biotinylated goat anti-mouse IgG (secondary antibody) for 10 min at room temperature and rinsed with PBS-Tween 20 four times (a few seconds). The tissue sections were then incubated with streptavidin peroxidase for 10 min at room temperature and rinsed with PBS-Tween 20 4 times (a few seconds). Then, 2 µl DAB chromogen was added to 100 µl of DAB substrate and applied on the tissue sections for 10 min followed by rinsing with PBS-Tween 20 four times (a few seconds). The tissue sections were observed under an inverted microscope using different magnifications (4×, 10× and 20×). Representative images of tissue sections processed using conventional IHC is shown in **Fig S4**.

Multiplexed µIHC using the vMFP.

In µIHC, dewaxing, rehydration, and protein block steps were performed according to the conventional IHC procedure. To implement multiplexed IHC, multiple syringes are used, each filled with a single antibody solution. Once processing of the tissue section with one

antibody is completed, the tubing between the vMFP head and the syringe is unscrewed and connected to another syringe. The tissue sections were placed in PBS-Tween 20 and then positioned on the vMFP platform. Fluorescein at a concentration of 5 µM was added to the primary antibodies to visualize the interaction of the confined liquid with the tissue section. Spots or trajectories were processed on the tissue sections by programming the head to be stationary at a specific location on the tissues for a fixed duration, or migrating the head along a trajectory at a specific velocity. In addition, adjacent spots on the tissue sections were created without the need to lift the head or stop the flow of fluid. This movement of the head, which we term "stop-and-go", required the head to migrate at a velocity greater than 3 mm s^{-1}. At these lateral velocities, a layer of the immersion liquid formed between the apex of the head and the tissue section and resulted in loss of contact between the confined liquid and tissue section[12]. For incubation of multiple antibodies, the syringes filled with antibodies were serially connected to the injection flow path of the vMFP.

For µIHC we set operating conditions of the vMFP such that the sheer forces exerted by the liquid were not disruptive to the tissue. These conditions were established experimentally and found compatible for a range of tissue sections. The conditions used were similar to that in our earlier work (Ref. 17) and consistent with analytical models in Ref. 21. Occasionally, commercially available tissue sections partially dislodged during pre-processing (de-waxing, antigen retrieval etc.). The use of such tissue sections is problematic and was avoided.

The thickness of the tissue sections used was 4 µm. Tissue sections are normally 3-7 µm thick, allowing brightfield transmission microscopy. In our work, we made use of such "transparent" tissue sections. Although thicker tissue sections are not routinely used, nor can they be optically visualized with a standard microscope, they are compatible for use with the vMFP. The head of the vMFP was mounted on a z-stage having sub-micrometer precision. It thus allowed for operation with different thickness of tissue sections, limited only by the length of the z-stage.

Data analysis of stained tissue sections.

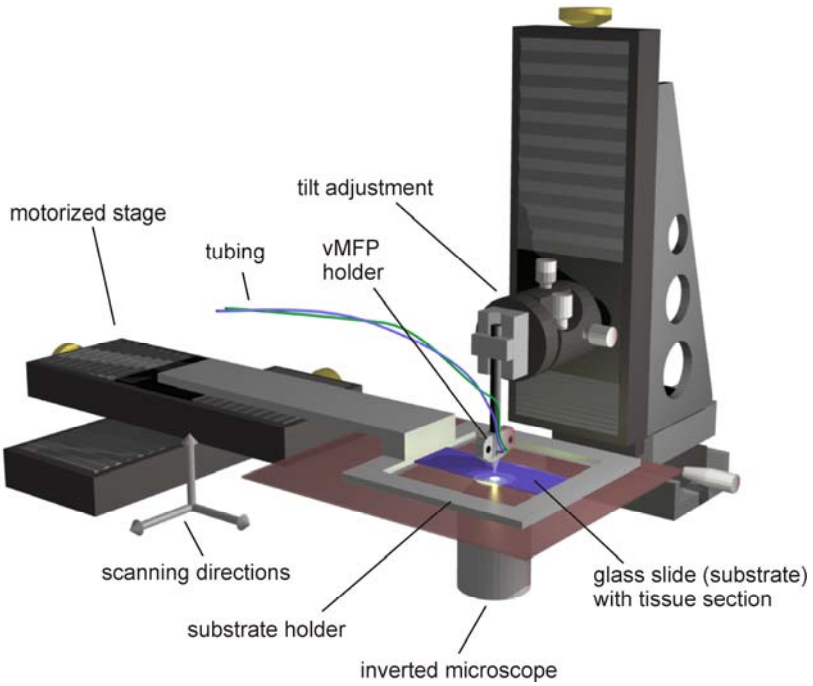
After µIHC, the tissue section was visualized in bright field using an upright microscope (×10 objective) and the images recorded on a computer. Analysis was performed on the images using a software developed in LabView 2010. In this software, the images were converted to 8-bit grayscale format and regions of interest (ROI) on the images were interactively selected. At least 15 ROIs for each residence time (0 s, 1 s, 2 s, 5, s 10 s, 20 s, 25 s, 50 s and 100 s) were chosen, mean intensities over all pixels calculated, background subtraction performed and the data recorded. The uncertainty bars in Fig. 2b were drawn to represent 95% confidence on the mean pixel intensities based on 15 ROIs for each residence time and the

curve fit was with a 2nd order butterworth filter with a normalized frequency of 0.4 (normalized to the Nyquist frequency using the mean intensities of the ROIs).

Flow confinement stability analysis.

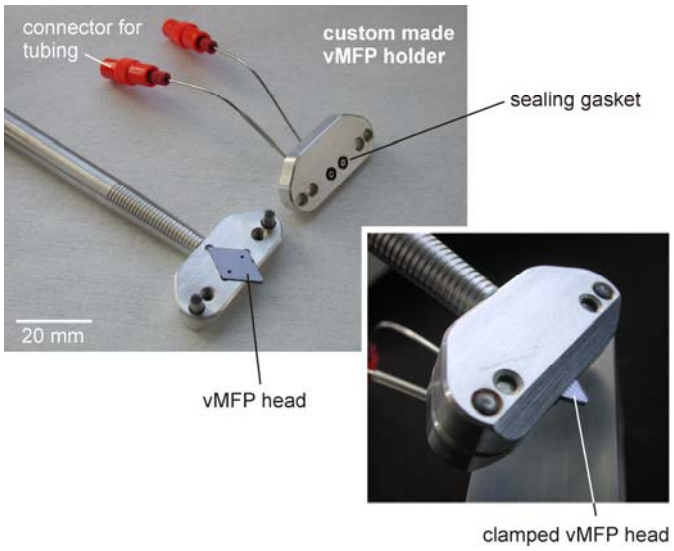
The stability of the flow confinement was measured by positioning the vMFP head 20 µm above a normal human thyroid tissue section or a glass surface. A confinement with fluorescein as the processing liquid was recorded using a video camera and analyzed using a software developed in LabView 2010. A threshold on the pixel intensity was interactively defined to establish the outline of the flow confinement. The variation of the flow confinement from the mean area was represented as a histogram.

Figure S1



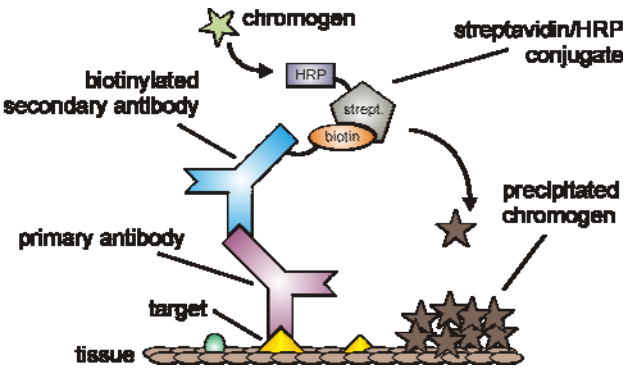
The vMFP platform had two linear motorized units that controlled the X and Y movement of the substrate holder and a linear control unit for the Z movement of the vMFP head. A custom-made holder for the vMFP head was mounted to a tilt adjustment unit to level the vMFP head precisely with the substrate (glass slide / tissue section). An inverted microscope served as the base of the platform and was used for the visualization of the confined liquid at the apex of the vMFP head. Precision syringe pumps were connected to the vMFP head mounted in the holder. The X, Y and Z movements as well as the syringe pumps were controlled through a graphical user interface developed in LabView 2010.

Figure S2



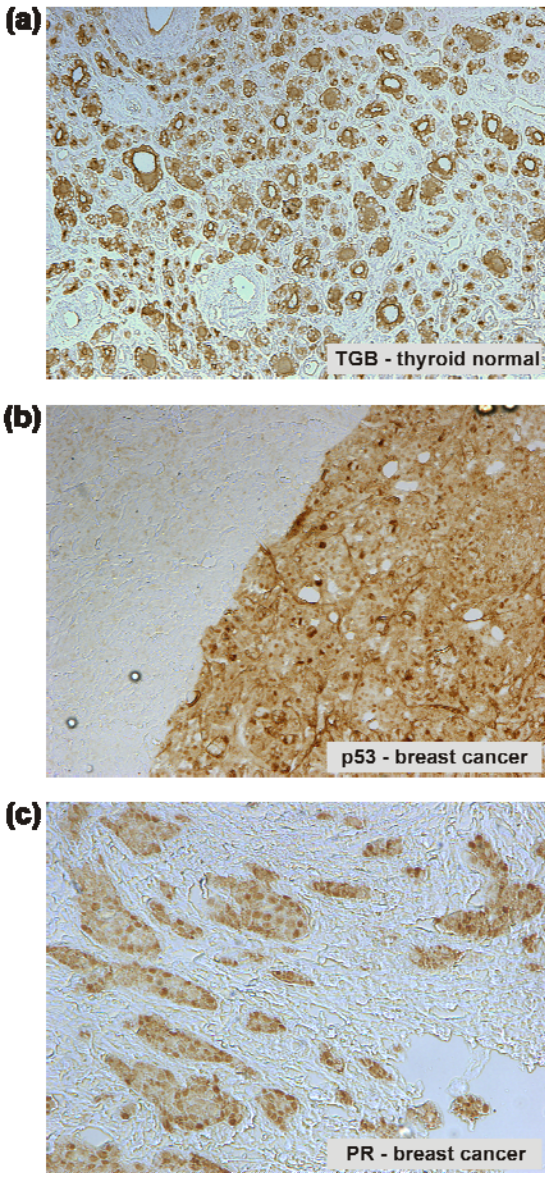
The vMFP head was mounted within a custom aluminum holder prior to performing an experiment. The head holder is shown here with a vMFP head before and after assembly. The syringe pumps (not shown) were connected to the holder through capillary tubing. O-rings were used to seal the connection between the vias of the vMFP head and the ports in the holder. If required, replacement and leveling of the vMFP head over a sample/tissue section was done within minutes.

Figure S3



A standard IHC method was used for the detection/visualization of the target (i.e. antigens) molecules. Primary antibodies directed against the target molecules (e.g. p53) were applied to the tissue section. These antibodies were selected depending on the type of tissue used for the experiment. A commercially available kit for enzyme mediated chromogenic detection (ab64259, Abcam, Cambridge, UK) was used for post-processing of the tissue section. This involved incubation with a biotin labeled secondary antibody and application of a streptavidin/horseradish peroxidase complex. Incubation with the chromogen 3,3'-diaminobenzidine (DAB) yielded a brown clearly visible product in the presence of the peroxidase.

Figure S4



Micrographs (10×) of conventionally IHC stained normal human thyroid tissue (a) and breast cancer tissues (b and c). Staining was performed using a standard IHC protocol similar to the protocol used for µIHC. The data was used to make assumptions on the required residence/incubation time of the primary antibodies applied with the vMFP. Low expression levels (signal) of markers determined in conventional IHC predicted longer residence times for vMFP based µIHC.