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Supporting Information

Monitoring Tyrosinase Expression in Non-metastatic and Metastatic Melanoma Tissues by Scanning Electrochemical Microscopy

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List of supplementary information:

■ SI-1. Optical microscopic images of normal skin tissue samples immunostained for tyrosinase detection

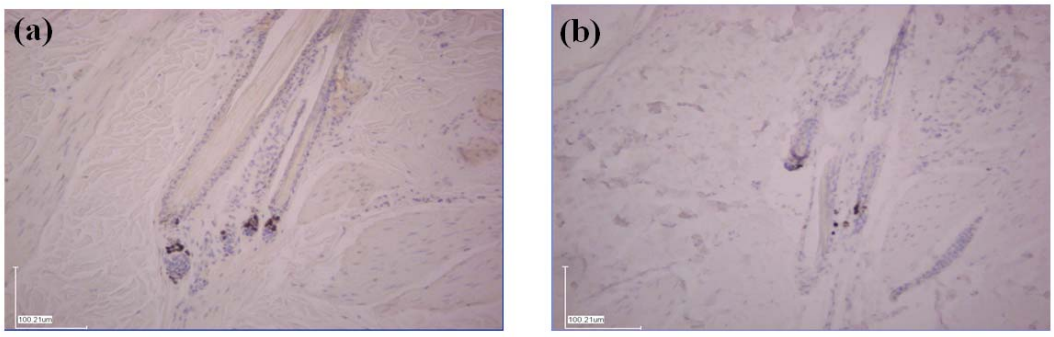

Figure S1. a) Optical microscopic image of an immunostained normal skin tissue for tyrosinase detection. b) Optical microscopic image of a normal skin tissue without tyrosinase immunostaining showing the presence of melanin.

3,3'-diaminobenzidine (DAB) is a common chromogen widely used in immunohistochemistry. Figure S1 demonstrates the similarity between DAB and melanin leading to an inference in the optical detection when applying immunostaining.

The following protocol was carried out for these two pictures. Normal skin tissues were de-paraffinized and rehydrated (details in SI-2). In Figure S1a, tyrosinase was labeled with primary antibodies (Abs) and horseradish peroxidase (HRP)-conjugated secondary Abs. In Figure S1b, no antibodies were applied. Then both tissue sections were washed with PBS buffer solution. Nuclear fast red (NFR) commercial solution was added in order to stain the nuclei. The tissues were all covered by NFR and stained for 5 min. Afterwards, the tissues were fixed within 10 min in 4% (v/v) paraformaldehyde (PFA) at room temperature (RT). The tissues were dehydrated quickly in EtOH 70% (v/v), EtOH 96% (v/v), three times in EtOH 100% (v/v) and xylene. Subsequently, the samples were covered with a proper cover slip. The brown color in Figure S1b resulted exclusively from melanin, because no immunostaining has been performed. Therefore, the brown color in Figure S1a cannot exclusively be adjudicated to the presence of tyrosinase, but also to the melanin coloration. The latter represents a serious interference in the diagnosis of tissue sections based on optical techniques.

■ SI-2. Experimental section

Chemicals

Bovine serum albumin (BSA), potassium dihydrogen phosphate, potassium monohydrogen phosphate, citric acid, xylene, ethanol, Triton X-100, tris base, 3,3',5,5'-tetramethylbenzidine (TMB), 3,3'-diaminobenzidine (DAB) and hydrogen peroxide (3 %) were bought from Sigma–Aldrich (Schnelldorf, Switzerland). Methanol was purchased from Merck (Dietikon, Switzerland) and formaldehyde solution (4% in PBS) was from Alfa Aesar (Karlsruhe, Germany). Anti-tyrosinase monoclonal antibody (Ab) T311 was obtained from Bio Medical, USA. Secondary anti-mouse Abs conjugated with horse radish peroxidase (Abs-HRP) were from Abcam, UK. Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3indolyphosphate (BCIP) were purchased from Roche, Switzerland. Deionized water was produced by a Milli-Q plus 185 model from Millipore (Zug, Switzerland). All reagents and materials were of analytical grade and used as received.

PBS buffer (10 mM, pH 7.4) was composed of NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM and KH_2PO_4 1.8 mM. Experimental, washing and blocking buffers of 1 % (v/v) BSA were prepared in PBS buffer. The substrate solution for the enzymatic reaction during SECM experiments was made in 0.1 M citrate-phosphate buffer, pH = 5.0, and contained additionally TMB and H_2O_2 (*vide infra*). Paraffin-embedded skin tissue microarrays (TMA) were from US Biomax, Inc. (U.S.A). One TMA contained sectioned skin biopsies of malignant melanoma and normal skin from three different patients (Figure S2-1a) and was used for the experiments shown in Figure 1a. Another TMA (Figure S2-1b) contained biopsies from another six different patients and was used for the experiments shown in Figure 1b, 1c and Figure 2. As noted by Biomax, the tissue sections were extracted from the patients' skin of arms, foot and chest wall.

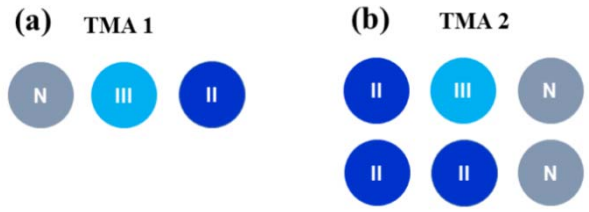

Figure S2-1. a) TMA 1 contained sectioned skin biopsies of malignant melanoma and normal skin from three different patients, and was used in Figure 1a. b) TMA 2 contained biopsies from another six different patients and was used in Figure 1b, 1c and Figure 2.

Protocol of conventional IHC method

Before the immunoassay, the tissue slices were de-paraffinized by immersing them twice in xylene for 10 min and rehydrated by immersing them sequentially in solutions of ethanol in water (100%, 95%, 70% and 30% of ethanol) (v/v) for 5 min each. Antigen retrieval was done in 10 mM citrate buffer, 95 °C, 20 min, followed by a cooling down to room temperature (RT) for 15 min. Finally, the tissue slices were incubated for 5 min in PBS buffer. The endogenous peroxidase activity was blocked by 10 min incubation of the samples in 3% (v/v) H_2O_2 in PBS at RT. Subsequently, primary Abs and HRP conjugated secondary Abs were applied. Then the tissue slices were equilibrated in alkaline phosphatase (AP) buffer (pH 9.5) which was composed of 0.1 M Tris base, 0.1 M sodium chloride, and 50 mM magnesium chloride. The tissue sections were covered with nitro blue tetrazolium (NBT) 0.03% (v/v) and 5-bromo-4-chloro-3-indoly phosphate (BCIP) 0.02% (v/v) in AP buffer for 2 h. The tissues were washed with PBS solution and then nuclear fast red (NFR) commercial solution was added in order to stain the nuclei. The tissues were all covered by NFR and stained for 5 min. Subsequently, they were fixed for 10 min in 4% (v/v) PFA at RT. A quick dehydration was performed in EtOH 70% (v/v), EtOH 96% (v/v), 3× EtOH 100% (v/v) and xylene. The samples were then covered with proper cover slips.

Protocol for the immunodetection of TyR in tissues. Before the immunoassay, the tissue slices were de-paraffinized by immersing them twice in xylene for 10 minutes and rehydrated by immersing them sequentially in solutions of ethanol in water (100%, 95%, 70% and 30% of ethanol) (v/v) for 5 min each. Antigen retrieval was done in 10 mM citrate buffer, 95 °C, 20 min, and followed by cooling at room temperature for 15 min. Finally, the tissues were incubated for 5 min in PBS buffer. The endogenous peroxidase activity was blocked by 10 min incubation of the samples in 3% (v/v) H_2O_2 in PBS at RT. The blocking solution was applied for 1 h at RT, and the residual fluid was completely removed. Thereafter, the solution of primary Abs against TyR (100 times diluted in PBS) was added to the samples, incubated for 1 h at RT and washed three times with PBS by placing the tissue in the buffer for 5 min. Finally, the slices were immersed in Abs-HRP (100 times diluted in PBS), incubated for 1 h at RT and washed with PBS as in the previous step. The tissue sections were placed in an electrochemical cell and the presence of TyR was established by SECM using the sample generation-tip collection mode by the detection of TMB_{ox} generated enzymatically at Abs-HRP (*vide infra*).

Soft stylus probes manufacturing

Soft stylus probes were manufactured by UV-photoablation of a microchannel inside 100 µm thick polyethylene terephthalate sheets (PET, Dupont, Wilmington, DE, USA), using a 193 nm ArF excimer laser beam (Lambda Physik, Göttingen, Germany, frequency 50 Hz, energy 250 mJ) as it was reported in detail previously.[1] The depth and the width of the microchannel were 20 µm and 30 µm, respectively. Subsequently, the microchannel was manually filled with a conductive carbon ink (Electra Polymer and Chemicals Ltd., Roughway Mill, Dunk Green, England), and cured at 80 °C. A 2 µm thick Parylene C layer was applied using a parylene depositioning system (Comelec SA, La Chaux-de-Fonds, Switzerland) to create the carbon ME by the exposure of the cross-section with the a razor or scalpel blade. Prior to each SECM experiment, the soft stylus probe was cut manually with a surgical scalpel blade (Swan-Morton, Sheffield, England) to obtain on the one hand a fresh electrode surface and to achieve on the other hand a V-shaped tip at the probe with an electrode area of 986 μm^2 (0.5 – 2 mm wide cross-section of the electrode body including the carbon ME). The probes were characterized optically and electrochemically by using optical microscopy and cyclic voltammetry, respectively. In SECM experiments, the Parylene C coating was facing the substrate surface and the Parylene C layer provided the constant distance between carbon ME and substrate (Figure S2-2). Figure S2-3 shows optical micrographs of the employed soft probe.

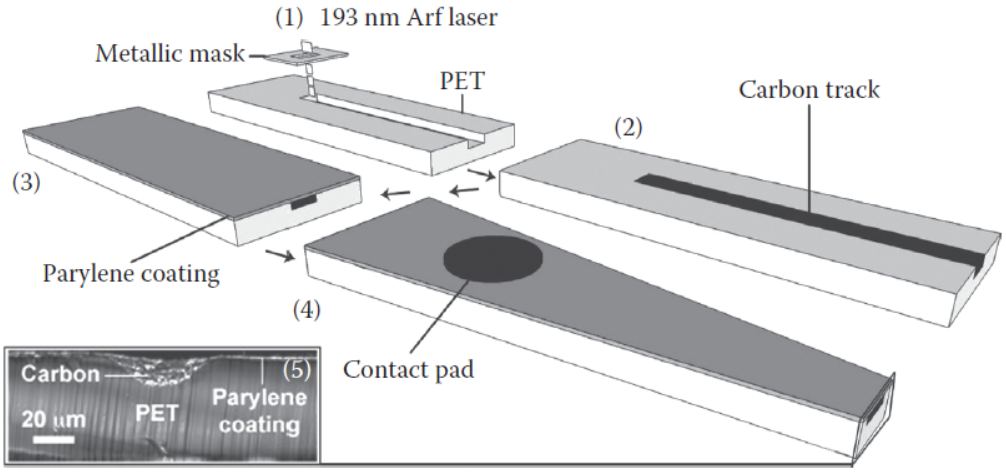

Figure S2-2. Soft stylus probes manufacturing: 1) UV laser ablation to fabricate a microchannel in a 100 µm thin polyethylene terephthalate (PET) sheet, 2) filling of the microchannel with a carbon paste followed by a curing process at 80 °C, 3) covering and sealing of the conductive carbon track with a Parylene C coating. 4) Shaping of the probe body and preparation of the electrical connection pad. From Bard, A. J., Cortés-Salazar, F., Girault, H.H.: Additional Recent Applications and Prospects in Scanning Electrochemical Microscopy, 2nd Edition. Copyright 2012 CRC Press. Reproduced with permission.

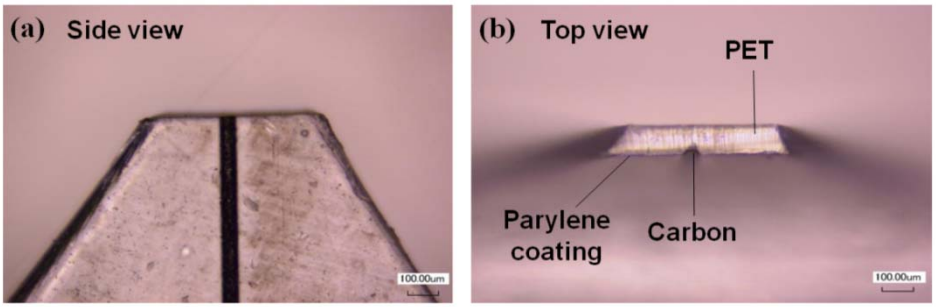

Figure S2-3. Photographs of soft probe from side (a), and top (b). The carbon ME, parylene coating and PET sheet are indicated.

SECM measurements

A custom-built SECM setup running under SECMx software (Gunther Wittstock, Carl von Ossietzky University of Oldenburg, Germany)[2] and equipped with a CompactStat (Ivium Technologies, Netherlands) and an electronic sample tilt table from Zaber Technologies, Canada, was used. All SECM experiments were performed in a three-electrode arrangement using a Ag wire as quasi-reference (QRE) and a Pt wire as counter electrode (CE). All potentials reported herein are given with respect to the Ag QRE. Measured data sets were processed and analyzed by using the software MIRA (Gunther Wittstock, Carl von Ossietzky University of Oldenburg, Germany).[3]

SECM images of TyR in tissues

For the SECM imaging of TyR in tissue sections, both the contact-less (*i.e.* constant height mode) and the contact mode (*i.e.* a probe brushing over the surface while keeping *d* constant) were employed.

Contact-less mode SECM experiments were performed with a conventional Pt microelectrode embedded in glass. Approach curves were performed over the insulating glass surface to measure the hindered diffusion current of the redox active species TMB. By this procedure, the sample tilt was eliminated and the working distance between glass surface and tip was set to 20 µm. Before SECM imaging, several test line scans were performed to ensure the working distance was larger than the thickness of the tissue. In case the thickness of the tissue slice appeared larger, *d* was adjusted.

Contact mode SECM experiments were performed using a soft stylus probe made of a carbon ME embedded in two polymer films (*vide supra*). The probe was fixed in a

special home-made holder to provide a 70° inclination angle between the substrate and the carbon ME to control the probe bending direction. For details, see reference [4]. The probe was brought into mechanical contact with the sample surface until h_p = –50 µm where h_p represents the vertical probe displacement after mechanical contact that the probe would make when it would penetrate into the substrate. For the definition of h_p, please see Figure S2-4. Approach curves were performed over the insulating glass surface to record the hindered diffusion current of the redox active species TMB. A lift-off routine included into the SECMx software was employed to perform the SECM images. Briefly, the soft stylus probe was kept in contact with the sample surface during forward line scans, in which current values were recorded, while it was operated in a contact-less regime (*i.e.* 100 µm tip-to-substrate distance) during the relocation to the starting position of the following forward line scan. In this way, overbending and additional pressure on the probe as well as on the substrate were avoided.

In the beginning of the HRP catalyzed enzymatic reaction, the concentration of TMB_{ox} was low and SECM signals were weak and less reproducible. Therefore in all experiments, special care was taken that the response from the substrate was stable during the time scale (15 min of equilibration) of the experiments in terms of steady-state diffusion layers from the active sites of the tissues.

Experimental conditions for the contact-less mode in Figures 1a, and Figure S5: working electrode Pt, r_T (radius of metallic disk) =12.5 µm, *RG* (ratio of r_T and insulating glass sheath) = 8-10, E_T (potential at ME) = –0.15 V, translation speed = 20 µm/s, d = 20 µm, delay of ADC time (time between probe translation was stopped and data acquisition was started) = 0.1 s. Therefore the convective disturbances induced by the probe translation can be negligible. 50 µM TMB and 25 µM H_2O_2 mixture in 10 mM citrate phosphate buffer (pH 5) was used as solution. For the reaction scheme see Figure S2-5.

Experimental conditions for contact mode: working electrode = carbon soft stylus, E_T = –0.15 V, translation speed = 50 µm/s, h_P = –50 µm, delay of ADC = 0.1 s. 50 µM TMB and 25 µM H_2O_2 mixture in 10 mM citrate phosphate buffer (pH 5) was used as solution.

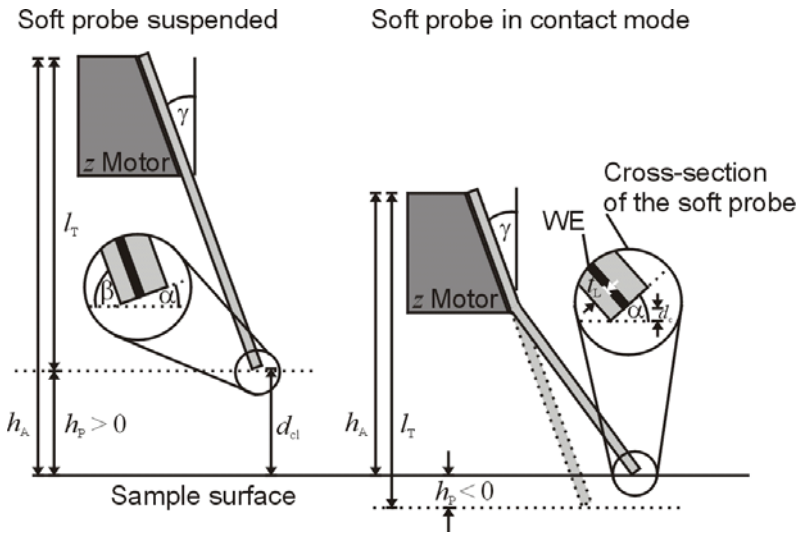

Figure S2-4. The h_P value is defined as the difference between the attachment point of the probe to the positioning system (h_A) and the length of the unbent probe (l_T) (*i.e.* $h_P = h_A - l_T$). h_P represents the working distance given by the positioning system that in contrast to the commonly used working distance d, can take negative values describing further probe-sample contact and d that would be achieved in case the probe would penetrate the sample. From Lesch, A., Vaske, B., Meiners, F., Momotenko, D., Cortés-Salazar, F., Girault, H.H., Wittstock, G.: Parallel Imaging and Template-Free Patterning of Self-Assembled Monolayers with Soft Linear Microelectrode Arrays. *Angew. Chem. Int. Ed.* 2012. 41. 10413–10416. Copyright 2012 Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced and modified with permission.

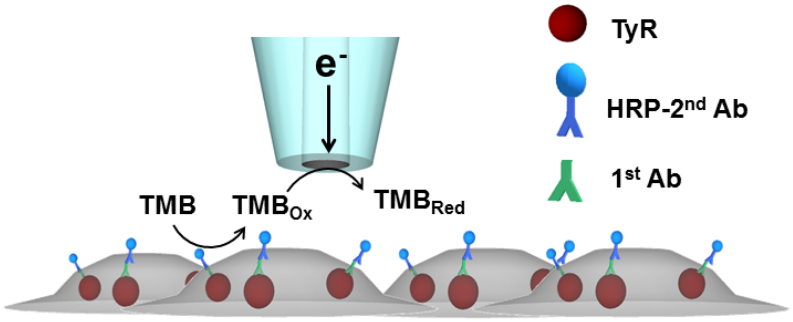

Figure S2-5. Schematic representation of the applied immunoassay strategy for the SECM sample generation/tip collection mode using the contactless SECM mode with a conventional ME. The strategy is equal to the contact mode apart from the employed probe.

■ SI-3. Tissue microarray (TMA) and SECM line scan directions

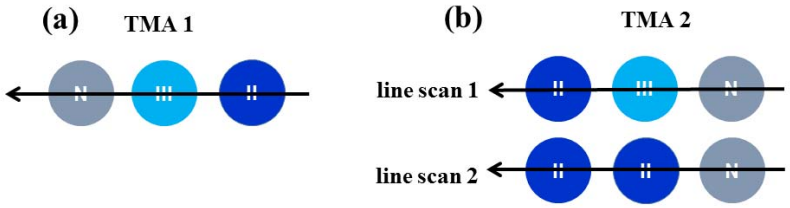

Figure S3. a) The different tissue sections in the TMA and line scan directions in the performed line scan experiment using the Pt ME in contact-less mode. b) The tissues in the TMA and line scan directions when using the carbon-based soft probe in contact mode.

The directions of the line scans are displayed in Figure S3. The line scans were performed as close to the centers of the tissue sections as possible. However, the TMAs were made by hand, and each tissue was slightly different in terms of shape and exact position. Therefore, the center of each tissue could slightly deviate from the center of the rows and lines, respectively.

In Figure S3a, the line scan passed over three skin biopsies from three different patients. In Figure S3b, two line scans passed over six tissues from another six different patients.

- **SI-4 Topography of the tissues before and after hydration**

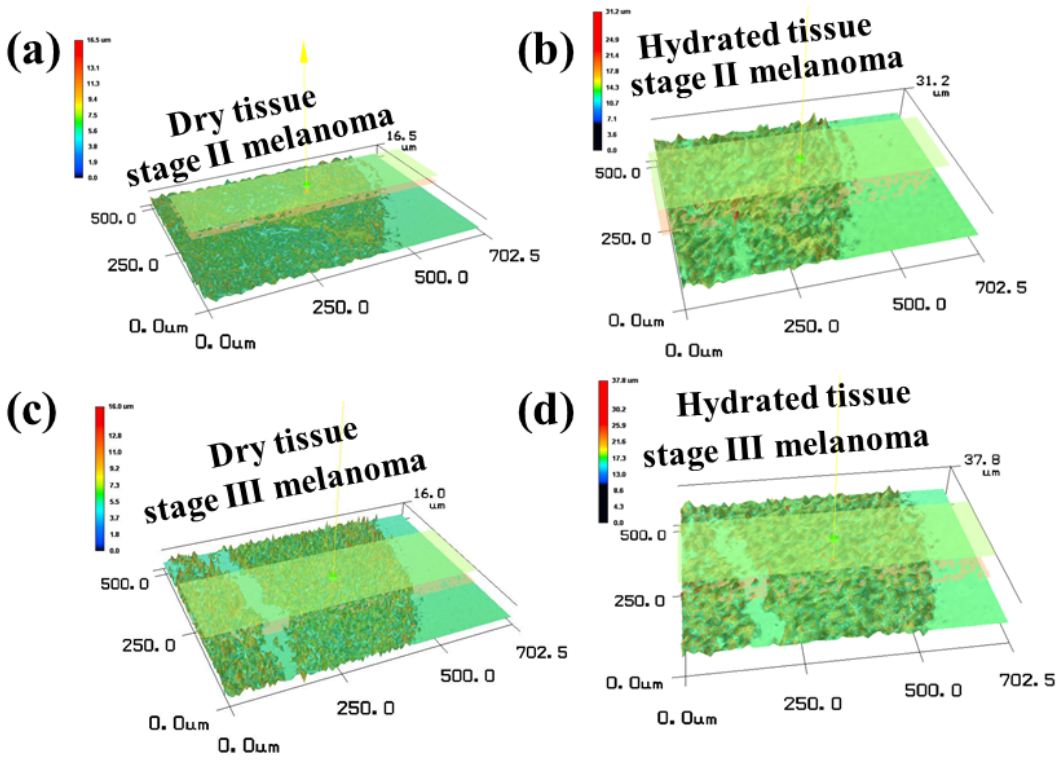

Figure S4. Topographic changes of stage II and stage III melanoma skin biopsies before and after hydration. a) Dry tissue section of stage II melanoma. b) Hydrated tissue section of (a). c) Dry tissue section of stage III melanoma. d) Hydrated tissue section of (c).

In Figure S4a, the average height deference between the stage II melanoma tissue and the glass surface is 8.7 µm. However, after the tissue is hydrated, the average height is 18.3 µm, demonstrating an increase of the tissue thickness of more than 100 %. Similarly, in Figure S4b, the average height deference between the stage II melanoma tissue and glass surface is 10.1 µm. After hydration, the height is 20.9 µm. This explains that the hydration condition can change the height of the tissue. Therefore, it is difficult to keep the same working distance in the traditional contact-less, constant height SECM mode. On the contrary, employing soft probes in contact mode enables scanning during which the topography of the swelling tissues is followed providing reliable information of the tissue reactivity.

- **SI-5. SECM images of immunostained tissues for tyrosinase detection in contact-less mode**

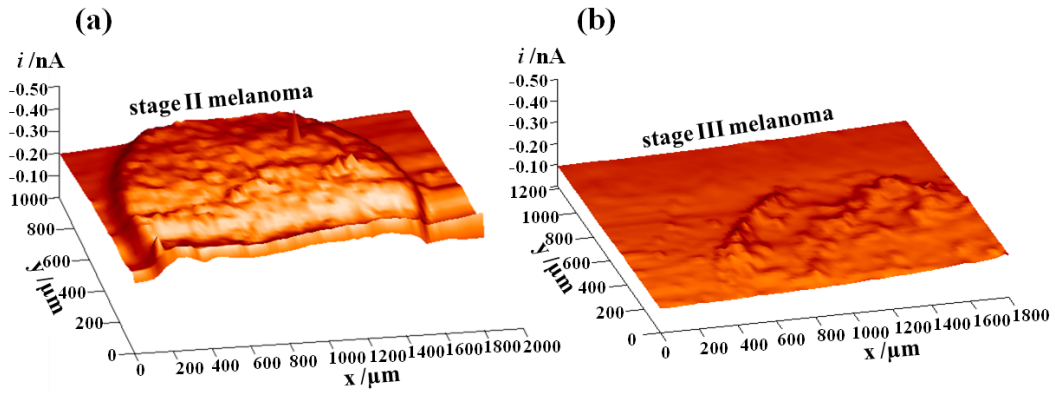

Figure S5. Constant height mode SECM image of stage II (a) and stage III (b) melanoma tissues.

Figure S5 shows SECM images of melanoma stage II and III samples recorded in the contact-less mode using a Pt ME. Although higher currents and a more homogeneous pattern were observed over stage II melanoma, it has to be made clear that topographic artifacts cannot be excluded. As it can be seen in the figures and discussions in the main manuscript, only by using soft probes for constant distance mode scanning of the tissues can provide reliable data.

■ SI-6 Optical micrograph of a tissue sample scratched with a ME during an SECM experiment

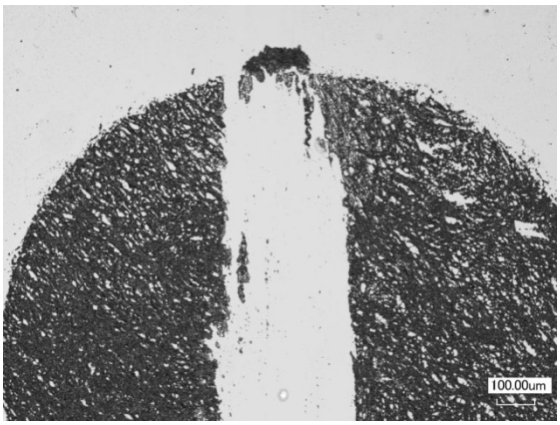

Figure S6. Optical micrograph of a micro-scratched tissue section after several repetitions of an SECM line scan using a conventional Pt ME enclosed in an insulating glass sheath.

The thickness of each tissue slice changes slightly depending on the tissue properties, the hydration level, tissue sectioning procedure, and general quality of the sample. Therefore, the tissue slice can be seriously damaged during SECM experiments when the ME gets in mechanical contact with the tissue. Figure S6 shows the scratch of a melanoma tissue caused by mechanical sample-probe contact. It is important to note that the ME was not in contact with the glass substrate, but with the tissue. The working distance d towards the glass was equal to 5 µm and the ME had a radius of the Pt disk equal to 12.5 µm with a radius of the insulating glass sheath of 250 µm. The uncertainty of the sample surface cannot only lead to the destruction of the sample but also to a pollution or damage of the probe. These issues can be overcome by employing soft probes.

■ SI-7. Picture of TMB precipitates on TMA

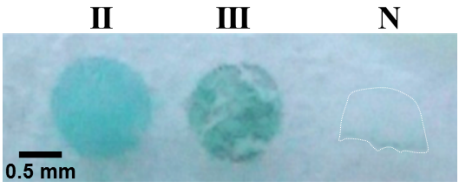

Figure S7. Photograph of a TMA containing stage II and stage III melanoma as well as normal skin after the line scan experiments shown in Figure 1b of the main manuscript.

After ten line scans performed with a soft probe in contact mode, the tissues remained well attached and intact (Figure S7). The blue color was from TMB_{ox} precipitated on the tissue sections after long term exposure to the assay. The location of TMB_{ox} precipitation has a strong correlation with the TyR distribution, which is in good agreement with line scan results in Figure 1b in the main manuscript. However, the original color of stage III melanoma was dark brown. Therefore, the color in the photograph is strongly interfered by its original color. In some cases, the melanin in stage II melanoma can cause serious interferences in the colorimetric detection. This shows another time the limitation of optical measurements.

■ SI-8. Negative control experiment to exclude non-specific binding

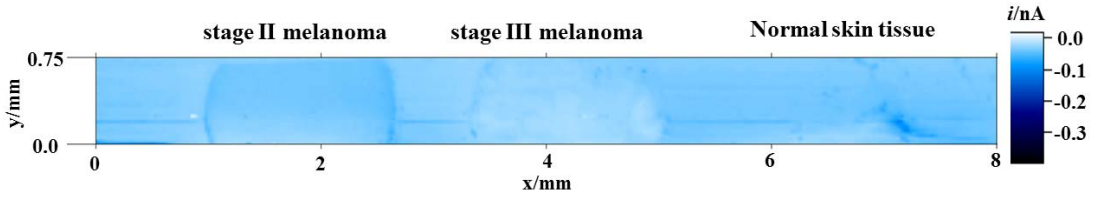

Figure S8. SECM image in contact mode of stage II, and stage III melanoma tissue, and normal skin sections without applying the primary Abs. Experimental conditions were the same as in Figure 2.

Figure S8 shows an SECM image of tissue sections without employing the preparation step of adding primary antibodies. Thus, this experiment is a negative control for non-specific binding of the HRP-labeled secondary Abs. The tissue sections were prepared as mentioned above. The sample was treated with the same immunoassay but in the absence of primary Abs. The recorded current values were significantly smaller and the current over the three tissues showed nearly the same level.

■ SI-9. Data extraction points from Figure 2

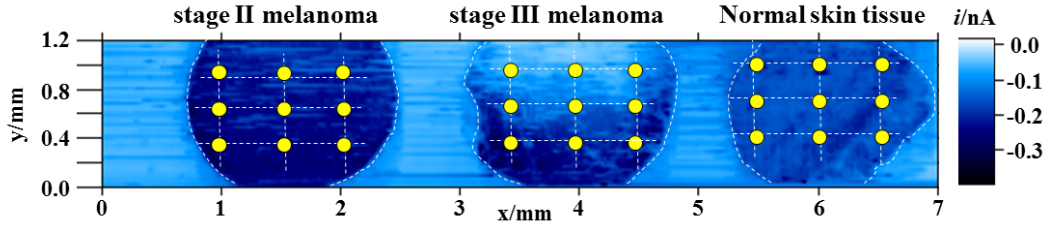

Figure S9. Illustration of the nine locations (as yellow spots) per tissue section from where the currents were extracted and averaged.

The currents were extracted every 0.5 mm in *x* direction and every 0.4 mm in *y* direction. The three tissue sections were intact and the area of tissue material is indicated by dashed lines. The averaged current values are shown in Figure 2 in the main manuscript.

■ SI-10. Definitions of cancer progression stages

Melanoma cancer stage definition: Melanoma can be classified in different stages according to their size, spatial and vertical growth and degree of spreading. Suggested by the American Joint Commission on Cancer (AJCC), consists of five stages: i) non-invasive and non-metastatic melanoma, (stage 0); ii) the tumor is non-metastatic and does not penetrate into dermis (stage I), iii) the tumor is non-metastatic and penetrates into dermis (stage II), iv) the tumor produce regional lymph node metastasis (stage III) and v) the tumor spreads across the whole organism (stage IV).[5]

References

[1] a) F. Cortés-Salazar, M. Trauble, F. Li, J.-M. Busnel, A.-L. Gassner, M. Hojeij, G. Wittstock, H. H. Girault, *Anal. Biochem.* **2009**, *81*, 6889-6896; b) F. Cortés-Salazar, A. Lesch, D. Momotenko, J.-M. Busnel, G. Wittstock, H. H. Girault, *Anal. Methods* **2010**, *2*, 817.

[2] C. Nunes-Kirchner, K. H. Hallmeier, R. Szargan, T. Raschke, C. Radehaus, G. Wittstock, *Electroanalysis* **2007**, *19*, 1023-1031.

[3] G. Wittstock, T. Asmus, T. Wilhelm, *Fresenius. J. Anal. Chem.* **2000**, *367*, 346-351.

[4] F. Cortés-Salazar, D. Momotenko, A. Lesch, G. Wittstock, H. H. Girault, *Anal. Chem.* **2010**, *82*, 10037-10044.

[5] S. B. Edge, C. C. Compton, *Ann. Surg. Oncol.* **2010**, *17*, 1471-1474.