Direct Chemosensitivity Monitoring Ex Vivo on Undissociated Melanoma Tumor Tissue by Impedance Spectroscopy

Heinz-Georg Jahnke1, Sarah Poenick1, Jan Maschke2, Michael Kendler2, Jan C. Simon2, and Andrea A. Robitzki1

Abstract
Stage III/IV melanoma remains incurable in most cases due to chemotherapeutic resistance. Thus, predicting and monitoring chemotherapeutic responses in this setting offer great interest. To overcome limitations of existing assays in evaluating the chemosensitivity of dissociated tumor cells, we developed a label-free monitoring system to directly analyze the chemosensitivity of undissociated tumor tissue. Using a preparation of tumor micro-fragments (TMF) established from melanoma biopsies, we characterized the tissue organization and biomarker expression by immunocytochemistry. Robust generation of TMF was established successfully and demonstrated on a broad range of primary melanoma tumors and tumor metastases. Organization and biomarker expression within the TMF were highly comparable with tumor tissue, in contrast to dissociated, cultivated tumor cells. Using isolated TMF, sensitivity to six clinically relevant chemotherapeutic drugs (dacarbazine, doxorubicin, paclitaxel, cisplatin, gemcitabine, and treosulfan) was determined by impedance spectroscopy in combination with a unique microcavity array technology we developed. In parallel, comparative analyses were performed on monolayer tumor cell cultures. Lastly, we determined the efficacy of chemotherapeutic agents on TMF by impedance spectroscopy to obtain individual chemosensitivity patterns. Our results demonstrated nonpredictable differences in the reaction of tumor cells to chemotherapy in TMF by comparison with dissociated, cultivated tumor cells. Our direct impedimetric analysis of melanoma biopsies offers a direct ex vivo system to more reliably predict patient-specific chemosensitivity patterns and to monitor antitumor efficacy. Cancer Res; 74(22); 6408–18. ©2014 AACR.

Introduction
The number of patients suffering from malignant melanoma continues to rise in most countries, which causes a high mortality rate in a relatively young population under the age of 50 years. Patients with melanoma (15%–25%) develop metastases, either regional (stage III) or distant (stage IV), and the majority of whom will die. Despite all efforts, the mortality of melanoma has not changed over the last 20 years, and current median survival of stage IV patients is about 6 to 9 month and a 5-year survival state of 0.5 % (1). Although novel therapeutic approaches either targeting melanoma-specific mutations or stimulating anti-melanoma immunity have recently been introduced into clinical care and more are in development; chemotherapeutics like dacarbazine and cisplatin are widely in clinical use. Nevertheless, there is the common consensus that there will be no unique single therapy for melanoma, instead personalized therapies based on patient and tumor-specific characteristics will be the future direction.

In this context, in vitro chemosensitivity assays are promising tools to predict the individual outcome of chemotherapeutics and, moreover, could be used for an easy and fast therapy control. Although previously developed chemosensitivity assays like the colony-forming unit assay, proliferation assay, and the MTT assay showed limited usefulness (2), the ATP assay (ATP-TCA) revealed promising results at least for the prediction of resistance for ovarian cancer (3) but limited for the prediction of sensitivity (4, 5). For other tumor entities like mamma carcinoma and melanoma, the ATP-TCA assay also revealed limited prediction capabilities (6, 7). Especially for melanoma studies, it was revealed to be inapplicable for certain drugs like dacarbazine or temozolomide (8). The major drawback of all the established chemosensitivity assays is the need of an extended amount of tissue (> 1 cm²) for quantitative analysis of at least 4 to 6 compounds (9). Moreover, the tumor tissue has to be completely dissociated, followed by further selection, filtering, and recultivation steps, resulting in a complete loss of
the original tissue organization, the extracellular matrix, and probably of whole cell populations (6). Especially in the context of tissue organization, recent studies demonstrated that the chemotherapeutic response pattern can be substantially altered in two-dimensional (2D) cultures (10–13). Therefore, there is a substantial demand for systems that can perform quantitative chemotherapeutics efficacy monitoring on three-dimensional (3D) cultures. Although molecular biologic end-point assays on 3D cultures are in general more laborious and/or can be easily falsified when the read-out (e.g., absorbance or fluorescence) is performed directly on the 3D culture, label-free bioelectronic methods have recent advantages. Especially, impedance spectroscopy is a suitable technique for characterization of single tumor cells (14) as well as cellular degradation in 2D cultures (15, 16) and, more strikingly, for quantitative monitoring of chemotherapeutics efficacy in 3D cultures (17, 18). Using our unique microcavity array (MCA) technology, we could detect the sensitivity or even an aggressive response to certain chemotherapeutics within 3D in vitro tumor models (19). So the important next step was to adapt the system for bioelectronic samples obtained from tumor biopsy material and demonstrate the applicability of the system for clinical use.

Materials and Methods
Tumor specimens and processing of melanoma tissue

Viable tumor tissue was obtained from tumor lesions after surgical removal of cutaneous or subcutaneous melanoma metastases and primary tumors. Therapeutically planned excision of the metastases was indicated by the referring clinicians independently of the possible additional use of left-over tumor specimens for our ex vivo and in vitro applications. Written informed consent according to the Declaration of Helsinki with local ethics committee approval was always obtained from each patient before surgery and before subjecting parts of the melanoma tissue to ex vivo chemosensitivity testing. All tumor tissues used in our assays were regarded as dispensable by the responsible clinicians and pathologists. Sterile tumor specimens were placed into 10-mL vials containing RPMI basal medium (Life Technologies) and processed within 4 hours. Fat and surrounding connective tissue was extensively removed by scalpel and tumor micro-fragments (TMF) prepared by manual cutting of tumor tissue to the appropriate size. TMFs were cultivated in 48-well plates (Greiner BioOne) with 300 μL complete growth medium (RPMI, 10 % FCS, 1 % glutamax, 0.2 % penicill/ streptomycin—all from Life Technologies) for 24 to 48 hours on a self-developed gyratory shaker (72 rpm). For isolation of tumor cells, the tumor tissue was minced using scalpels and seeded into T125 culture flasks in 1-mL complete growth medium. After 2 to 3 days, medium was exchanged, and adhered cells were expanded for experiments performed on monolayer cultures within five to ten passages. For immunocytochemical staining, tumor tissue pieces were fixed in 4 % formalin (Merck) for 60 minutes, preserved in 25 % sucrose solution (Carl Roth), and cryodissected to 20-μm slices by a CM 3050S cryostat (Leica).

Chemotherapeutic agents, vital staining, and immunocytochemistry

Dacarbazine, doxorubicin, paclitaxel, and cisplatin were purchased from Sigma-Aldrich, gemcitabine from Enzo Life-science, and treosulfan from the pharmacy at the University of Leipzig Medical Center (Leipzig Germany). Microscopic images were taken with an inverse Nikon TE2000 microscope. For vital staining, cells were incubated with 2.5 μmol/L Calcein-AM (Life Technologies) and 1 μmol/L propidium iodide (Sigma-Aldrich) for 10 minutes and imaged with a Nikon TE2000 microscope.

For immunocytochemical staining, fixed and cryosegmented slides were permeabilized and blocked with 3 % BSA containing 0.1 % Triton-PBS for 45 minutes at room temperature (all from Sigma-Aldrich). Afterwards, the sections were incubated with anti-HMB45 (1:100; Dako), anti-S100 (1:200; Dako), anti-MelanA (1:1000; Abcam), E-cadherin (1:100; Cell Signaling Technology), MelCAM (1:100; Santa Cruz Biotechnology), and collagen I (1:200; Abcam) for two hours followed by incubation with DyLight-488- or DyLight-549-conjugated anti-rabbit or anti-mouse secondary antibodies for 90 minutes (Dianova). Finally, nuclei were stained by incubation with DAPI (1 μg/ml) for one minute, followed by washing, drying, and covering with Kaiser’s glycerol gelatin (Merck). Images were taken using a Nikon C1 plus confocal microscope (TE2000).

Electrochemical impedance spectroscopy

Impedometric measurements on 2D cultures were performed as previously described (20). Briefly, 5 × 104 melanoma cells were seeded in 200-μL complete growth medium per well on self-developed interdigital electrode (IDE) arrays coated with collagen I (Life Technologies) and cultivated for 3 to 4 days until cell layer confluence. Impedance spectra (500 Hz to 5 MHz, 51 points, 10 mV amplitude) were automatically recorded every 30 minutes for 96 hours with our impedance measurement platform based on an Agilent 4294A high-precision impedance analyzer (Agilent Technologies) and our self-developed controlling software IMAT v2.2.1. After 1 hour premonitoring, the experiment was started by the application of chemotherapeutics at the described concentrations. For control groups, the appropriate solvent was used. For measurements on TMFs, we used our self-developed MCA (21) with pyramidal cavities (edge length 400 μm) in combination with the same impedance measurement platform as used for the IDE arrays. Impedance spectra (5 kHz to 5 MHz, 51 points, 100 mV amplitude) were recorded. After the initial measurement (0 hour), the TMFs were individually transferred to 48-well plates containing the concentration of the tested chemotherapeutics and incubated on the gyratory shaker for 72 hours. For discrete time points, TMFs were transferred manually to the MCA (18), impedance spectra were recorded instantly (approx. 5 seconds per TMF), and TMFs were transferred back to the gyratory shaker. For data analysis, our self-developed software IDAT v3.6 was used to automatically extract the cellular contribution to the impedance magnitude by calculating the relative impedance according to the equation (∣Zw| with cells − |Z| without cells)/Z without cells × 100 % and, afterward, tracing the
maximum cell signal (peak) over time. For each independent experiment on IDE arrays, four replicates (wells) were used per treatment. For each independent experiment on TMF, six TMFs were randomly selected and analyzed individually per treatment. For statistical analysis, the relative impedance maxima were normalized to experimental start (0 hour) and to control values.

**XTT assay**

Metabolic activity and thereby cell viability were quantified using an *In Vitro Toxicology Assay Kit*, based on the tetrazolium salt XTT (Sigma-Aldrich). Therefore, melanoma cells (4 × 10⁵ cells/well) were seeded onto adherent 96-well plates. After incubation with compounds for 72 hours, the XTT solution was added for 3 hours, and the absorbance was measured at 450 nm with a Sunrise plate reader (Tecan). In addition, the absorbance at 690 nm was measured as a background and subtracted from the 450 nm value as well as the blank (in accordance to the manual). All values of an independent experiment were normalized to the control values.

**ATP assay**

Cell viability was quantified using the ATPlite assay (PerkinElmer) according to the manufacturer’s instructions. Therefore, melanoma cells (4 × 10⁵ cells/well) were seeded onto adherent 96-well ViewPlates (PerkinElmer) in a volume of 100 μL. After incubation with compounds for 72 hours, 50 μL of cell lysis solution was added and incubated on an orbital shaker (700 rpm) for five minutes. Addition of 50 μL substrate solution (Luciferase/Luciferin) and shaking for five minutes initiated the reaction for quantifying ATP concentration. After covering the bottom with an adhesive seal and 10-minute dark adaption, luminescence was measured using an Infinite 200 luminescence plate reader (Tecan). The blank (without cells) was subtracted, and the ATP concentration was calculated according to a standard curve.

**Statistical analysis**

All statistical analyses were performed using Graphpad Prism 5.02. All presented graphs are based on independent experiments (number described by “n = ” in each graph). Presented values are given as mean (±) SEM until otherwise described. IC₅₀ values were determined by nonlinear sigmoidal curve fitting with the normalized response and constant slope setting. TMF-derived IC₅₀ values are presented with 95 % confidence intervals (CI) and IC₁₀ values from independent experiments as mean (±) SEM. Multiple group comparisons were done by the two-way ANOVA and Bonferroni *post hoc* test. Differences between two means with *, *P* < 0.05 were considered significant; **, *P* < 0.01 very significant; and ***, *P* < 0.001 extremely significant.

**Results**

**Establishment of TMF-based impedimetric monitoring system**

Because there was no label-free real-time monitoring system for the reliable and quantitative chemotherapeutics efficacy monitoring on 3D cultures available, we developed the MCA technology as previously described (19). For application of this system to tumor biopsy material, the first challenge was the reliable generation of 3D tissue pieces with a certain size and sufficient number. The main prerequisite was to use a minimum of tumor biopsy material and the conservation of the tumor cell composition and organization. Therefore, enzymatic digestion and reaggregation steps were excluded. Finally, our approach was the manual sectioning of the tumor tissue using scalpel, stereomicroscope, and clean bench for obtaining TMFs (Fig. 1A) for the impedimetric monitoring using our unique MCAs (Fig. 1B). We were able to easily generate TMF of distinct sizes ranging from 200 to 1,000 μm in diameter. After initial trials for evaluating optimum TMF size and yield, we identified TMF with an average diameter of 500 μm as optimum allowing us to isolate at least 300 TMFs from only 0.15 cm³ of solid tumor tissue. In a next step, we tried to optimize the irregular shape of the TMF. Moreover, although commonly the TMF core showed vitality directly after preparation, the outer shell consisted of damaged cells caused by the cutting (Fig. 1C). Both problems could be minimized by initial culturing on a gyratory shaker (Fig. 1B) for at least 24 to 48 hours (Fig. 1C). It has to be mentioned that optical methods like the fluorescence-based live/dead staining could not be performed on all tumor biopsies. Especially, tumor material with high melanin concentrations (black) showed quenching and absorbance effects mainly in the green and red spectral range (Supplementary Fig. S1). Finally, we were able to generate rounded and overall vital TMF from a broad range of melanoma primary tumors as well as metastases (Fig. 1C, right), including solid but also metastases with necrotic and ambiguous cores. Generated TMF revealed an average diameter of 400 μm. Therefore, we used MCA with a cavity size of 400 μm (Fig. 1B). Taken together, we established a robust protocol for generation of TMF that could be used for the MCA-based bioelectric analysis within 2 days after preparation (Fig. 1A).

**TMF reflects organization and tumor marker expression of native tumor tissue**

After successful preparation and generation of TMF from tumor biopsies, we questioned how the structural organization, composition, and tumor marker expression of the TMF compare with native tumor tissue and with cells derived from dissociated tumor tissue. Therefore, we obtained from one solid melanoma metastasis samples for fixation and direct immunocytochemical staining of native tissue, TMF, and monolayer cultures (Fig. 2). First, we investigated the three commonly used melanoma markers HMB45, S100, and MelanA (MLANA). Although direct comparison of the staining intensity has to be done carefully because of the different material processing (fixation, cryodissociation for the tissue, TMF, etc.), the native tumor tissue and TMF show high similarity, whereas there are clear differences in the distribution of the tumor markers in monolayer cultures. Especially, HMB45 showed more homogeneous and higher expression in monolayer cultures. The same could be observed for S100 and MelanA that were more widely expressed in native tumor tissue and TMF.
Taken together, the tumor tissue and TMF showed a more heterogeneous distribution of the tumor markers. Moreover, staining of the extracellular matrix component collagen I (COL1A) clearly showed comparable amount and distribution in the tumor tissue and TMF in contrast to the monolayer cultures where only residual collagen I could be observed. Furthermore, native tumor tissue and TMF showed high level of E-cadherin (CDH1) expression in nearly all cells and only residual MelCAM (MCAM) expression, whereas monolayer cultures showed high and widely MelCAM expression but only low E-cadherin expression.

Taken together, the immunocytochemical analysis revealed a high comparability in tissue architecture, tumor marker, and extracellular matrix components expression in native tumor tissue and TMF but not in monolayer cultures.

**Impedimetric characterization and chemosensitivity monitoring of TMFs**

In a next step, we wanted to characterize the TMF by impedance spectroscopy. Therefore, we characterized a range of primary melanomas and melanoma metastases for cell density and distribution. Exemplarily, we show the detailed analysis of a primary tumor that showed low cell density but homogenous cell distribution, a tumor metastasis with high cell density, and a tumor metastasis with overall moderate cell density but heterogeneous distributed high cell density clusters (Fig. 3A). Because tumor tissue biopsies were obtained from different patients, the extracted cellular contribution to the impedance magnitude spectra (relative impedance) revealed substantial differences (Fig. 3B). Although at lower frequency (5 kHz) no differences were observed, the relative impedance maximum of the primary tumor was significantly lower when compared with the metastases (4.5 % vs. 7.3 % and 7.1 %). Furthermore, the frequency of the maximum impedance was significantly different in all cases. More interestingly, metastasis 1 (TM1) showed a significant higher relative impedance at 5 MHz. For a better understanding of how the observed spectra differences correlate with cellular characteristics, we established an equivalent circuit model for the TMF-MCA system that is dominated by the cellular capacitance (cell membrane capacitance), the cellular resistance (cell membrane resistance), and the intercellular resistance (Supplementary Fig. S2A). The mathematically simplified equivalent circuit model (Supplementary Fig. S2B) allows the easy visualization of the influence of each parameter (Supplementary Fig. S2C).
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Although an increased cell membrane capacitance leads to a lowered impedance maximum shifted to lower frequencies, the increase of the cell membrane resistance leads to an increased impedance maximum also shifted to lower frequencies. More interestingly, the increase of the intercellular resistance leads to a substantially increased impedance value at 5 MHz. On the basis of the proposed model, this means for tumor metastasis 2 that treatment with cisplatin leads to an increased cell density over the whole TMFs. For more details, we analyzed the relative impedance maximum at concentrations of 3, 10, and 100 μmol/L cisplatin in a time-dependent manner (Fig. 3E). While for the primary tumor 10 μmol/L cisplatin already induced a significant decrease to 63% after 48 hours that was further decreased to 56% after 72 hours, tumor metastasis 1 showed an initial significant increase within the first 8 hours to 139% for 3 μmol/L and 126% for 100 μmol/L followed by a significant decrease to 55% only for 100 μmol/L after 72 hours. In previous studies on dissociated tumor cells using the ATP-TCA assay, sensitivity to cisplatin was assigned to a concentration level below 1 to 10 μmol/L or lower concentrations within 48 hours and as insensitive when a significant impedance decrease was observed at 10 μmol/L. In accordance to this definition and our observations on TMF measurements, we assigned a tumor as sensitive when a significant impedance decrease was observed within 24 hours (8, 22). In contrast, melanoma marker MelanA was highly expressed in monolayer cell cultures but nearly completely absent in the biopsy and biopsy-derived fragments (blue, cell nuclei). Bar, 50 μm.

In the TMF is lowered. Furthermore, a low relative impedance value at 5 MHz can be assigned to a lowered extracellular resistance, which means there are less cell–cell contacts or even bigger extracellular spaces between the cells. So the combination of a low maximum and a low 5 MHz relative impedance value can be correlated with a TMF that contains low numbers of cells that show overall relative big extracellular spaces between the cells. If both relative impedance values are shifted to higher values, this can be correlated with a higher number of cells in a TMF and overall, small extracellular spaces and distinct cell–cell contacts. The third case is represented by a high relative impedance maximum and a low relative impedance value at 5 MHz that can be correlated with a higher number of cells in the electrical field but with certain extracellular spaces between the cells or cell clusters. The immunocytochemical stainings of the three investigated tumors (Fig. 3A) correlate with our proposed model. Next, we tested the effect of cisplatin on the isolated TMF. The detailed impedance spectra analysis after 72 hours incubation with 100 μmol/L cisplatin revealed individual effects for all three presented examples (Fig. 3D). Although for the primary tumor and tumor metastasis 1–derived TMF there was a significant decrease of the relative impedance maximum, tumor metastasis 2 showed a significant increase in the relative impedance maximum and relative impedance at 5 MHz. On the basis of the proposed model, this means for tumor metastasis 2 that treatment with cisplatin leads to an increased cell density over the whole TMFs. For more details, we analyzed the relative impedance maximum at concentrations of 3, 10, and 100 μmol/L cisplatin in a time-dependent manner (Fig. 3E). While for the primary tumor 10 μmol/L cisplatin already induced a significant decrease to 63% after 48 hours that was further decreased to 56% after 72 hours, tumor metastasis 1 showed an initial significant increase within the first 8 hours to 139% for 3 μmol/L and 126% for 100 μmol/L followed by a significant decrease to 55% only for 100 μmol/L after 72 hours. In previous studies on dissociated tumor cells using the ATP-TCA assay, sensitivity to cisplatin was assigned to a concentration level below 1 to 10 μmol/L within 24 hours (8, 22). In accordance to this definition and our observations on TMF measurements, we assigned a tumor as sensitive when a significant impedance decrease was observed within 24 hours (8, 22). In contrast, tumor metastasis 2 showed a concentration- and time-dependent impedance increase with a maximum of 211% for 100 μmol/L cisplatin after 72 hours. Taking our cellular distribution model (Fig. 3C) into account as well as a prior impedimetric study on 3D cultures (18), the impedance increase can be correlated with a higher cell density over the whole TMF. That means the analyzed tumor metastasis 2 can be considered as aggressive to cisplatin because the increased cell density can be only caused by an increasing amount of tumor cells within the TMF.
After demonstrating that it is possible to quantitatively measure the effect of a certain chemotherapeutic on individual TMF, we wanted to use our impedimetric monitoring system to determine chemotherapeutic- and tumor-specific response patterns. Therefore, we tested six commonly used chemotherapeutics (dacarbazine, doxorubicin, paclitaxel, gemcitabine, cisplatin, and treosulfan) for melanoma. In general, we applied at least six concentrations ranging from 0.03 to 1,000 μmol/L.

The concentration range was adapted for each chemotherapeutic based on prior evaluations for in vitro chemosensitivity assays (22–24) as well as our own preliminary investigations on tumor cell line–derived spheroid cultures (19). For each tumor biopsy, we performed immunocytochemical characterization (Fig. 4A) to assure that the material analyzed was of melanoma origin and to get an overview of tissue organization, including extracellular matrix (collagen I), fibroblasts, and vessels visualized by CD90 (THY1). Because the impedimetric monitoring is a noninvasive and label-free method, the effect of the applied chemotherapeutic (exemplarily shown for cisplatin) can be monitored over time to retrieve a time- and concentration-dependent response pattern (n ≥ 4).

Figure 3. Impedimetric characterization of tumor fragments and determination of tumor-specific response to chemotherapeutics. A, exemplarily, a primary tumor and two tumor metastases were chosen that comprised a different expression of the tumor marker HMB45 (red) and cell density/distribution within the tumor tissue, visualized by the nuclei staining (blue). B, these individual tumor characteristics can be quantitatively analyzed by label-free impedance spectroscopy. The relative impedance spectra revealed tumor-dependent maximum at distinct frequencies as well as a tumor structure-dependent relative impedance value at 5 MHz (n ≥ 130 fragments, mean ± SD). C, on the basis of the equivalent circuit model described in Supplementary Data, a scheme for explanation of cell density/distribution within tumor fragments and correlation to the analyzed impedance parameter (relative impedance maximum and at 5 MHz) were proposed, where a low relative impedance MAX and 5 MHz correlated with a low number of cells and big extracellular spaces in a TMF, high relative impedance MAX and 5 MHz correlated with a high cell number and cell contacts in a TMF, and high relative impedance MAX in combination with a low 5 MHz value correlated with an higher cell number in the electrical field but with certain extracellular spaces between cells and cell clusters. D, the tumor-specific response to 100 μmol/L cisplatin was analyzed by impedance spectroscopy. The derived values for the 72-hour treatment were normalized to the appropriate controls (100%, dashed line) and revealed the tumor-specific effect of the applied cisplatin (n ≥ 4). E, because impedance spectroscopy is a noninvasive and label-free method, the effect of the applied chemotherapeutic (exemplarily shown for cisplatin) can be monitored over time to retrieve a time- and concentration-dependent response pattern (n ≥ 4).
concentration-dependent response pattern could be obtained for each tumor biopsy (Fig. 4C; Supplementary Figs. S3 and S4). Live/dead staining could be performed with limitation to melanin amount and distribution and correlated with the impedance spectroscopy–derived results. When impedance showed an early and substantial decrease like for doxorubicin, this correlates with low level of living cells and an extremely high amount of dead or damaged cells. In contrast, for paclitaxel, where no response to the chemotherapeutic could be observed by impedance spectroscopy, the live/dead staining revealed a high amount of living cells and nearly no damaged or dead cells. In general, it has to be mentioned that the fluorescence-based live/dead staining reflects more the outer shell of the TMF that is related to the amount and distribution of melanin that can quench the fluorescence signal. Only the impedimetric results reflect the status of the whole TMFs including the TMF core.

Because we were able to get more than 300 fragments from each clinical tumor specimen with at least 0.15 cm³, each treatment (at least 4 to 6 concentrations and controls) could be performed with six replicates. The replicates were randomly chosen from all fragments to take tumor heterogeneity into account. This allowed us to calculate concentration response curves and determine IC₅₀ values for responding TMF. The comparison of tumor metastases from three different patients revealed the unique time-dependent response pattern for each patient (Table 1). On the basis of the IC₅₀ values obtained over time on the same TMF, for each chemotherapeutic, a classification as sensitive, insensitive, and nonresponding could be proposed according to the definition described in the previous paragraph.

**Correlation of TMF- and monolayer cultures–derived chemosensitivity patterns**

Next, we wanted to compare the impedance spectroscopy response patterns from TMF with that obtained from dissociated and recultivated monolayer cultures. Moreover, we wanted to compare the results of our impedimetric analysis with established cytotoxicity (XTT assay) and chemosensitivity assays (ATP assay). Because both, the XTT- and ATP assay, can only be performed on monolayer cultures, the comparison of all three methods could only be done for these 2D cultures. For this experiment, we took a tumor metastasis, isolated the TMF, and dissociated the residual material (more than the half tumor biopsy). The dissociated cells were expanded so that for each assay on monolayer cultures three independent experiments could be performed. All comparisons were done based on the determined IC₅₀ values.
that were obtained for each individual experiment on monolayer cultures and averaged (Table 2). The significance analysis revealed little differences between the three methods, which were only temporal effects for doxorubicin (24 hours, impedance vs. XTT and ATP) and treosulfan (24 hours, each method against each other). For paclitaxel, this was more prominent (48 and 72 hours, impedance vs. XTT and ATP). It has to be mentioned that in contrast to the monolayer measurements that were repeated for each isolated cells and assay in three independent experiments, the measurement on TMF could only be performed one time because the TMFs of an individual tumor biopsy are only available once and could not be (sub)divided or further expanded. So the statistical reliability of the determined IC\textsubscript{50} values for the TMF measurements is given by the CI, in contrast to the mean ± SEM IC\textsubscript{50} values determined from three independent experiments on expanded monolayer cultures. Taking this into account, nevertheless, there were substantial differences between the IC\textsubscript{50} values on TMF and monolayer cultures by impedance spectroscopy. Despite gemcitabine, where no response could be detected overall, all other chemotherapeutics showed differences in the range of one decade and more. More strikingly, these differences did not tend to a single direction like for cisplatin, where the TMF showed no response or higher IC\textsubscript{50} values in comparison with the monolayer cultures. In contrast, doxorubicin and dacarbazine showed higher sensitivity in TMF than in monolayer cultures. Taken together, this demonstrates that a theoretical upscaling of results obtained from 2D cultures into a situation within a directly ex vivo-generated 3D tumor sample is not possible.

### Discussion

The aim of the presented work was to develop a method for feasible testing and monitoring of tumor sensitivity to chemotherapeutics from native tumor material. Although established molecular biologic assays based on dissociated and recultivated tumor cells showed predictive benefits for single tumor entities (4, 5), recent studies also demonstrated clear limitations of the predictive power for different tumor entities like the malignant melanoma (6). Moreover, actual studies revealed that 2D cultures show substantial alterations with regard to chemotherapeutic sensitivity when compared with 3D cultures (10–13) and that the tumor cell microenvironment like extracellular matrix components and individual cell–cell contacts are important for tumor biology as well as responsiveness to chemotherapeutics (25, 26). For this reason, we established a preparation method that does not destroy the original tumor organization and extracellular matrix. Specifically, we prepared TMFs from a range of primary tumors and tumor metastases of malignant melanoma. We could demonstrate that TMF highly represents the tumor metastases of malignant melanoma. We could demonstrate that TMF highly represents the tumor metastases of malignant melanoma.

### Table 1. Three patient–specific response patterns to the six investigated chemotherapeutics obtained by impedimetric analysis (4 ≤ n ≤ 6 TMF), expressed as IC\textsubscript{50} values in μmol/L (95% CI)

<table>
<thead>
<tr>
<th></th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
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<tbody>
<tr>
<td>TM 1</td>
<td></td>
<td></td>
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<tr>
<td>Dacarbazine</td>
<td>173.1 (19.3–1556) sensitive</td>
<td>94.8 (29.7–307.0) sensitive</td>
<td>93.8 (35.1–250.5) sensitive</td>
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<tr>
<td>Doxorubicin</td>
<td>187.1 (1.56–2535) insensitive</td>
<td>189.0 (0.9–2577) insensitive</td>
<td>189.0 (0.9–2577) insensitive</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>5.32) sensitive</td>
<td>20.6) sensitive</td>
<td>79.5 (33.8–187.1) sensitive</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>0.84) sensitive</td>
<td>11.8) sensitive</td>
<td>80.8 (32.4–202.0) sensitive</td>
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<tr>
<td>Cisplatin</td>
<td>3.10) sensitive</td>
<td>0.23 (0.0.9) sensitive</td>
<td>3.00 (32.4–202.0) sensitive</td>
</tr>
<tr>
<td>Treosulfan</td>
<td>4.38) sensitive</td>
<td>1.46 (0.68–7.56) sensitive</td>
<td>252.6 (68.4–933.2) sensitive</td>
</tr>
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<td>DMF</td>
<td></td>
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<tr>
<td>Dacarbazine</td>
<td>304 (127–727) sensitive</td>
<td>203.7 (83.5–497.0) sensitive</td>
<td>115.7 (61.1–219.3) sensitive</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>60.2 (14.4–147.6) sensitive</td>
<td>15.2 (11.2–20.6) sensitive</td>
<td>7.56 (4.86–11.8) sensitive</td>
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<tr>
<td>Paclitaxel</td>
<td>44.1 (14.4–135.2) sensitive</td>
<td>68.9 (15.0–316.1) sensitive</td>
<td>2.89 (1.57–5.32) sensitive</td>
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<td>Gemcitabine</td>
<td>3.46 (2.33–5.12) sensitive</td>
<td>4.39 (2.42–7.97) sensitive</td>
<td>1.82 (1.04–3.20) sensitive</td>
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<tr>
<td>Cisplatin</td>
<td>79.5 (33.8–187.1) sensitive</td>
<td>83.0 (252.6–88.9) sensitive</td>
<td>88.9 (38.4–205.6) sensitive</td>
</tr>
<tr>
<td>Treosulfan</td>
<td>252.6 (68.4–933.2) sensitive</td>
<td>115.7 (61.1–219.3) sensitive</td>
<td>115.7 (61.1–219.3) sensitive</td>
</tr>
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Table 2. Methodological comparison of the determined chemosensitivity patterns

<table>
<thead>
<tr>
<th>Impedance spectroscopy</th>
<th>Biopsy</th>
<th>Monolayer</th>
<th>XTT</th>
<th>ATP</th>
<th>Diff. 2D/3D</th>
<th>Diff. Meth.</th>
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<td>24 h</td>
<td></td>
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</tr>
<tr>
<td>Dacarbazine</td>
<td>—</td>
<td>—</td>
<td>256.2 (± 2.5)</td>
<td>9.41 (± 1.15)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1.33 (0.41–4.38)</td>
<td>—</td>
<td>—</td>
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<td></td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>—</td>
<td>353.7 (± 140.3)</td>
<td>256.2 (± 8.5)</td>
<td>357.5 (± 0.5)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>—</td>
<td>75.1 (± 16.0)</td>
<td>59.0 (± 8.1)</td>
<td>36.1 (± 3.1)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Treosulfan</td>
<td>—</td>
<td>194.3 (± 53.1)</td>
<td>—</td>
<td>632.8 (± 112.4)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>48 h</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Dacarbazine</td>
<td>167.1 (63.1–444.0)</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1.46 (0.68–3.10)</td>
<td>29.1 (± 13.1)</td>
<td>1.74 (± 0.13)</td>
<td>1.53 (± 0.22)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>—</td>
<td>206.7 (± 51.6)</td>
<td>38.3 (± 10.2)</td>
<td>86.7 (± 16.5)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>—</td>
<td>24.3 (± 6.8)</td>
<td>9.90 (± 0.55)</td>
<td>10.4 (± 0.4)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Treosulfan</td>
<td>—</td>
<td>170.9 (± 56.5)</td>
<td>140.4 (± 21.6)</td>
<td>204.1 (± 0.7)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dacarbazine</td>
<td>88.9 (38.4–205.6)</td>
<td>—</td>
<td>—</td>
<td>281.6 (± 48.2)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.23 (0.09–0.84)</td>
<td>1.89 (± 0.84)</td>
<td>0.74 (± 0.12)</td>
<td>0.53 (± 0.11)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>—</td>
<td>163.7 (± 62.4)</td>
<td>3.15 (± 1.82)</td>
<td>42.6 (± 7.4)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>102.6 (58.4–180.3)</td>
<td>16.1 (± 4.3)</td>
<td>7.46 (± 0.48)</td>
<td>7.38 (± 1.34)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Treosulfan</td>
<td>252.6 (68.4–933.2)</td>
<td>149.1 (± 61.7)</td>
<td>40.5 (± 9.0)</td>
<td>118.2 (± 5.7)</td>
<td></td>
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</tr>
</tbody>
</table>

NOTE: Impedimetric measurements on TMF (4 ≤ n ≤ 6; 95% CI) were compared with expanded tumor cells in 2D cultures that were analyzed by impedimetric measurement, XTT assay, and ATP assay (n = 3; SEM). For all assays, the response is expressed as IC50 value in μmol/L. Differences between 2D and 3D as well as the used methods are marked by X.

...and MelCAM. Our results are in line with previous comparative studies of in vivo situations as well as in vitro 2D cultures (27) and emphasize the importance of the tumor microenvironment. The efficacy of chemotherapeutics and novel therapeutic approaches considerably depends on the extracellular matrix components, their organization, adhesion molecules as well as cell–cell contacts (28, 29).

Therefore, we focused on the evaluation of optimized size for TMF. The generation of TMF was possible with only smaller amounts of tumor material (<0.15 cm³) in comparison with the amount that is needed for the ATP assay (>1 cm³; ref. 8). This is an important advantage because the tumor tissue amount needed for the ATP assay limits the clinical use for certain tumor entities with a low tumor volume like primary melanoma tumors (24). In addition, the dissociation and isolation of cells from tumor tissue can lead up to 75% of damaged cells (8) and, therefore, introducing the risk of selection of certain tumor or nontumor cells while eliminating others. Therefore, TMF-based chemosensitivity monitoring offers substantial advantages over monolayer culture–based methods. The main limiting step for TMF chemosensitivity monitoring is the manual preparation of the TMF from the tumor biopsy material. To overcome this limitation, we are working on automated dissection protocols so that this technique could be more easily expanded for, e.g., extended clinical studies.

The second aim of this work was to establish a method for the feasible monitoring of the TMF. Using our unique MCA technology, we applied impedance spectroscopy for the label-free and noninvasive characterization of the TMF composition. On the basis of the correlative immunocytochemical analysis, we established an equivalent circuit model allowing to retrieve more cell and tissue-specific information out of the complex impedance spectra (30, 31). So additional impedance spectra–derived parameters could be used for analyzing the heterogeneity of the isolated tumor tissue and included in the standard chemotherapeutic sensitivity analysis in the manner of an impedimetric multiparameter-derived principal component analysis. Therefore, we could demonstrate that impedance spectroscopy can be used for the reliable chemosensitivity measurement on native melanoma specimen similar to that obtained by XTT and ATP assays. Especially, the comparison to the widely used ATP assay (2) revealed only rare significant differences for the determined IC50 values. In contrast, the comparison between TMF and monolayer culture–derived IC50 values that were obtained by impedance spectroscopy showed substantial differences. More strikingly, these differences in our currently used direct ex vivo 3D system were not predictable in a direction like initial in vitro studies on cell line–based comparisons between 2D and 3D systems demonstrated previously (32). Although the demonstrated dataset only represents a single case, the detailed IC50 value–based...
statistical analysis demonstrates the applicability of impedance spectroscopy for the chemosensitivity monitoring of 2D and 3D cultures and, moreover, that results obtained from monolayer cultures cannot be extrapolated to 3D in vitro–like cultures. Of course the measurement of TMFs from an individual tumor biopsy can only be performed once, comparable with a histologic analysis of the tumor tissue itself. But the demonstrated time- and concentration-dependent analysis based on the amount of TMFs that can be obtained even from small biopsy samples allows for the calculation of time-dependent IC50 values with CIs that reveal a statistically more comprehensive sensitivity pattern than, e.g., for ATP-TCA–based end-point assays on monolayer cultures without any statistical reliability information (8, 22) or sum values with high variances (24). For the impedimetric quantification of TMF response to chemotherapeutics at least for sensitive tumors, the determined CIs are in the range of the CIs for TMF response to chemotherapeutics at least for sensitive micro heterogeneity of a single tumor is reflected in the heterogeneous response of the individual TMFs from the same tumor. This leads to higher variances of the determined impedance values and, finally, bigger CIs that correlate with a classification of an insensitive tumor.

Taken together, we established a novel system for the reliable and direct ex vivo chemotherapeutic sensitivity monitoring of tumor entities like melanoma. This system could be helpful, especially for tumor entities with poor clinical outcome where patient-specific chemotherapeutic treatment is required. We could demonstrate that chemosensitivity testing on TMF reveals unpredictable different results in comparison with dissociated and in vitro–recultivated tumor cells.

In conclusion, we have examined for the first time a new method for the quick and direct ex vivo chemosensitivity monitoring of native tissue from patients with melanoma. In a next step, our system should be validated in future studies where the predictive power will be correlated with the outcome of the chemotherapy for an appropriate number of patients. Given the high number of solid neoplasms from different organs, we want to point out that our system is not restricted to the malignant melanoma. Therefore, it should be tested on further tumor entities, especially where chemosensitivity assays on dissociated tumor cells show low predictive power.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H.-G. Jahnke, J.C. Simon, A.A. Robitzki
Development of methodology: H.-G. Jahnke, J. Maschke, A.A. Robitzki
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.-G. Jahnke, S. Poenick, M. Kendler, J.C. Simon
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): H.-G. Jahnke, S. Poenick, J.C. Simon
Writing, review, and/or revision of the manuscript: H.-G. Jahnke, S. Poenick, J.C. Simon
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Maschke, A.A. Robitzki
Study supervision: A.A. Robitzki

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References


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