

Dynamics of human cancer cell lines monitored by electrical and acoustic fluctuation analysis†

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Early determination of the metastatic potential of cancer cells is a crucial step for successful oncological treatment. Besides the remarkable progress in molecular genomics- or proteomics-based diagnostics, there is a great demand for *in vitro* biosensor devices that allow rapid and selective detection of the invasive properties of tumor cells. Here, the classical cancer cell motility *in vitro* assays for migration and invasion relying on Boyden chambers are compared to a real-time biosensor that analyzes the dynamic properties of adherent cells electro-acoustically with a time resolution on the order of seconds. The sensor relies on the well-established quartz crystal microbalance technique (QCM) that measures the shift in resonance frequency and damping of an oscillating quartz crystal when adsorption, desorption or changes in material properties close to the quartz surface occur. In addition, the QCM is capable of detecting the rather subtle fluctuations of the cell bodies as an indicator for their micromotility. QCM-based micromotility readings of three different cancer cell lines (HT-29, HSC-4, FaDu) are compared with the well-known electrical cell-substrate impedance sensing (ECIS) revealing collective stochastic motion that corresponds to the malignancy of the cells.

Introduction

Over the last half century, cancer and non-transferable cardiovascular diseases have replaced bacterial or viral infections as the major cause of death. Although, for instance in the US, cancer (22.8%) and heart diseases (26.6%) are still the leading causes of natural death, the number of cancer victims for the four most common cancers (prostate, breast, lung, colorectal)

have been declining since the early 1990s.¹ This positive trend is primarily due to a considerable progress in diagnostics and the availability of customized therapies. Breakthrough progress, however, is expected from new *in vitro* assays that sensitively detect the metastatic potential of tumor cells derived from a minimal amount of biopsy material.

Almost 90% of all tumors originate from epithelial tissues, since these are often directly exposed to carcinogenic substances. These cells may then spread from the site of primary transformation to other organs or non-adjacent sites via a cascade of events including invasion through a basement membrane, migration to the next blood vessel, intravasation, extravasation and eventually secondary tumor formation.² The metastatic potential of tumor cells is thought to be mirrored in their ability to invade and to migrate—or in more general terms: in their motility. Cell migration can be categorized into individual (amoeboid) or collective movement of cohesive multicellular units.^{3–6} Single-cell migration permits cells to cover local distances as found in neural crest cell migration. Individual migration modes are also held responsible for

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† Electronic supplementary information (ESI) available: Fig. S1 shows the typical dependency of a quartz resonator response on the seeded cell number of FaDu cells. Immediate confluency is reached at 500 000 cells. See DOI: 10.1039/b920815a

Insight, innovation, integration

As a response to the increasing demand for early determination of the metastatic potential of cancer cells we present a novel biosensor approach based on resonance frequency fluctuations of a thickness shear mode resonator to quantify cell motility of human cancer cells. Apart from monitoring attachment and spreading of anchorage-dependent cells with the quartz crystal microbalance this device is capable of

detecting the rather subtle fluctuations of the cell bodies as an indicator for their micromotility and biological activity. We found that the frequency fluctuations reproduce the metastatic potential of three cancer cell lines that were determined by conventional assays based on the well-established Boyden chamber. Interestingly, the cells display extremely long-memory stochastic noise distinct from thermal motion.

cancer metastasis at distant sites from the primary tumor.³ Collective migration plays an important role in formation and remodeling of tissue but also contributes to tumor progression by local invasion. The common process underlying all migration modes is polarized actomyosin driven change of the cell shape.^{4,7,8} Motility of cells is closely related to swift formation and annihilation of adhesion plaques. Weak adhesion of cells to the extracellular matrix (ECM) is equivalent with a high propensity for different modes of migration. Invasive properties of cells *in vitro* are most frequently quantified by means of so called Boyden chamber assays.⁹ These assays determine the amount of cells per time that migrate through a porous polycarbonate membrane with pores of a defined size (in the micrometre range), coated with a layer of ECM proteins. Various modifications of this original assay have been developed over the past decades. For instance, migration of cells through the pores of the membrane is quantified without any extracellular matrix coating on the surface of the membrane (migration),¹⁰ with a monolayer of endothelial cells (extravasation assay)¹¹ that the cells have to pass through or with an attractive or repellent gradient across the membrane (chemo- or haptotaxis assay).¹² Besides the Boyden chamber-based approaches, other assays have been used extensively in experimental oncology, *i.e.* sprouting assays,¹³ phagokinetic tracking,¹⁴ wound-healing assay¹⁵ and the cell scatter assay.¹⁰

Positive correlation between the metastatic potential as studied *in vivo* and these *in vitro* assays have been frequently demonstrated.¹⁶ Major drawbacks of these classical methods are the time and labor requirements and the material-intense data collection, often highly significant as they limit the number of experiments that are possible.

Requirements for up-to-date sensors of cellular motility comprise label-free and non-invasive monitoring of cellular activity in real time with a minimal number of cells. A reasonable time resolution will reveal even subtle changes of the cellular dynamics producing a broader parameter space than conventional endpoint assays. The statistical parameters allow correlation of cell migration spatially and temporally. Modern surface sensitive techniques such as impedimetric or acoustic sensors allow the measurement of subtle changes in cell shape as well as the cell substrate distance, important indicators for migratory propensity of cells.^{17–22} Recently, the quartz crystal microbalance (QCM) has gained a wide-spread interest as a label free, non-invasive biosensor to study biomedical applications and to follow adherent eukaryotic cells, also in cancer research.²³ A variety of different experiments have been described, for instance, the kinetics of cell attachment and spreading for different cell lines and seeding densities,²⁴ analysis of muscle-cell contraction or integrin-mediated adhesion of ovarian cells,^{25,26} monitoring the cell response

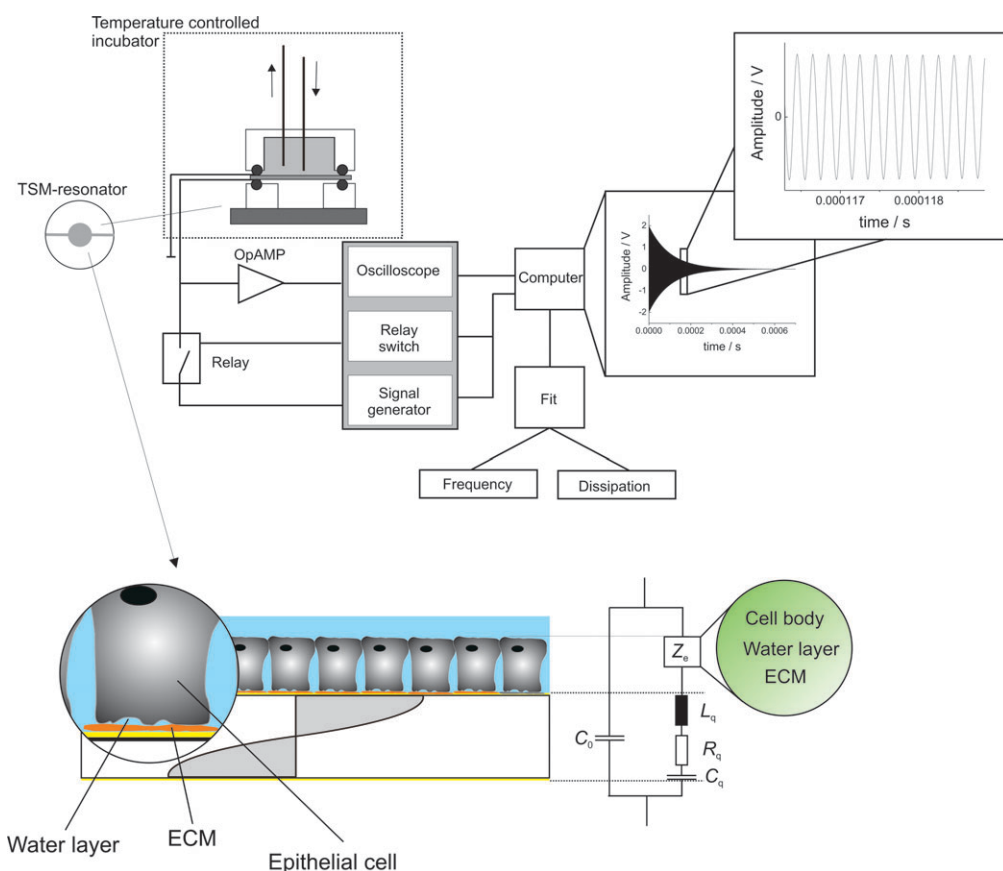


Fig. 1 Schematic illustration of the experimental D-QCM setup used in this study. The thickness shear mode resonator is driven by a signal generator and periodically disconnected. An operational amplifier feeds the signal into an A/D-card used to follow and store the free oscillation decay. The decay curves are subject to non-linear fitting which provides the resonance frequency and the decay constant (as a measure for energy dissipation) of the oscillation.

upon exposure to various chemotherapeutics or growth-factors^{27–30} or measuring the impact of nanoparticles on the viability of cultured epithelial cells.³¹

Here, we report on a QCM-based sensor that enables the study of collective cell motility of confluent cell monolayers represented by frequency fluctuations of a cell-covered quartz resonator (Fig. 1). These fluctuations of the resonance frequency report on the collective dynamics of both, cell shape variations and alterations of cellular micromechanics. Biological “noise” created by adherent cells displays not only the general activity of living systems, but also allows identifying signatures of long-memory correlations in motility as it becomes apparent from fluctuation analysis.^{19,32,33}

In a sense, QCM-based noise analysis of the resonance frequency is similar to the measurement of the so-called micromotion by an impedimetric whole cell biosensor referred to as electric cell–substrate impedance sensing or short ECIS.^{34–37} Recently, we have already reported on the general feasibility of fluctuation analysis as a powerful tool to measure cell motility in a technical paper.³⁸ Here, we compare the motility of three different confluent cancer cell lines measured by fluctuation analysis of the resonance frequency with classical Boyden chamber assays of migration and invasiveness as well as micromotion analysis of ECIS data. The cell lines FaDu, HT-29, HSC-4 are known to form confluent monolayers and show a rising invasiveness in the following order HSC-4 > HT-29 > FaDu. Variance analysis of the time series of the resonance frequency reproduces this sequence, while ECIS micromotion could not confirm the malignancy trend found in Boyden chamber assays.

Experimental section

Cell culture conditions

All human cancer cell lines used in this study originate either from colon or pharynx. FaDu cells,³⁹ a squamous carcinoma cell line derived from the hypopharynx, is cultured in Dulbecco's modified Eagles medium with 4.5 g/l glucose and 2 mM L-glutamine (PAA laboratories GmbH, Cölbe, Germany), non essential amino acids NEA (100 µg/ml), sodium pyruvate (1 mM) and 10 mM HEPES buffer. The colon carcinoma cell line HT-29⁴⁰ is cultured in DMEM low glucose (1.0 g/l), 1 mM Na-pyruvate and 1 mM L-glutamine (PAN, Aidenbach). The oral squamous epithelial carcinoma line HSC-4⁴¹ requires DMEM high glucose (4.5 g/l) with 2 mM L-glutamine (PAA, Cölbe), 1 mM HEPES buffer (PAA, Cölbe), 1 mM NEA (PAA, Cölbe) as well as 1 mM Na-pyruvate (PAA, Cölbe). Passage number is typically kept between 10 and 25 to ensure that the neoplastic phenotype did not change during the experiments.

Boyden chamber migration and invasion assays

Boyden chamber-based migration and invasion assays are carried out to confirm the known sequence of invasiveness of the three different cancer cell lines (HSC-4, HT-29 and FaDu). A 24-well Boyden chamber assay with inserts containing ECM-free or ECM-coated filter membranes was performed following the manufacturer's protocols (colorimetric ECMatrix

cell invasion assay, ECM 550, and colorimetric Chemotaxis cell migration assay, ECM 508, both from Chemicon, Temecula, CA, USA). Prior to seeding, the cells were pre-incubated in serum free-medium for 24 h. 2.1×10^5 cells suspended in 300 µl of their respective medium were loaded into the upper compartment of the Boyden chamber. Cells were incubated either for 72 h on ECM coated (invasion) or for 15 h on non-coated (migration) 8 µm-pore-sized polycarbonate membranes. Medium containing 10% (v/v) serum was filled into the lower chambers as a chemo-attractant source. Cells that had invaded through the matrix-covered or the uncoated pores of the membrane commonly adhere to the lower membrane surface of the filters and were subsequently quantified (after removal of non-invaded ones with cotton swabs from the upper surface) by using the cell staining solution from the Chemicon-kit. The numbers of migrated or invaded cells were determined by counting six fields of view at 40× magnification per well with a phase-contrast microscope (Axiovert 200, Zeiss, Germany) before averaging (see Fig. 2). Experiments were repeated three times.

QCM resonator preparation and phase contrast microscopy

100 nm thick circular gold electrodes (0.2 cm²) were thermally evaporated on both sides of 5 MHz AT-cut quartz crystals. 5 nm of chromium was used to ensure adhesion of gold. Optical microscopy is only possible with semi-transparent electrodes (12–50 nm gold on 3 nm chromium). Before use, the resonators are cleaned in an ultrasonic bath for 15 min at 70 °C. Afterwards, the crystals are dried under a stream of nitrogen and treated for 45 s in an argon plasma for sterilization and hydrophilisation to promote cell adhesion. Regeneration of sensors after the QCM experiments was accomplished by immersing them in a 70 : 30 ethanol–water mixture followed by a detergent washing step to remove the remaining cells from the surface before the cleaned resonators were rinsed three times with de-ionised water.

Special Teflon-chambers—used to form a culture vessel with the resonator at the bottom—are assembled aseptically and then filled with 1.5 ml of culture medium.²³ Sealing of the chamber is ensured by two o-rings clamping either side of the quartz. Finally the chambers are sealed with a screw cap to prevent fluid evaporation. The screw cap was equipped with an inlet and outlet to permit medium exchange. The measurement chambers are placed inside an ordinary cell culture incubator (Fig. 1) with 37 °C and 5% CO₂-atmosphere (MMM Medcenter Einrichtungen GmbH, Gräfelfing, Germany). After 2 h of equilibration, freshly passaged cells are directly transferred onto the resonator.

The cells were inoculated at a density of $6\text{--}7 \times 10^5$ cells/ml in aliquots up to 500 µl in (1 : 1) HEPES–HCO₃[–] buffered medium. As the resonator surface area is approximately 1 cm² and all cell types that are considered in this study are similar in size (mean area of the spread cell is 150–250 µm²). We adopted an experimental seeding density of $6\text{--}7 \times 10^5$ cells per cm² to ensure immediate and reproducible formation of a confluent monolayer accompanied by moderate medium consumption. Fig. S1† shows the frequency change as a function of seeded cell number. For each cell line, experiments are done in triplicate.

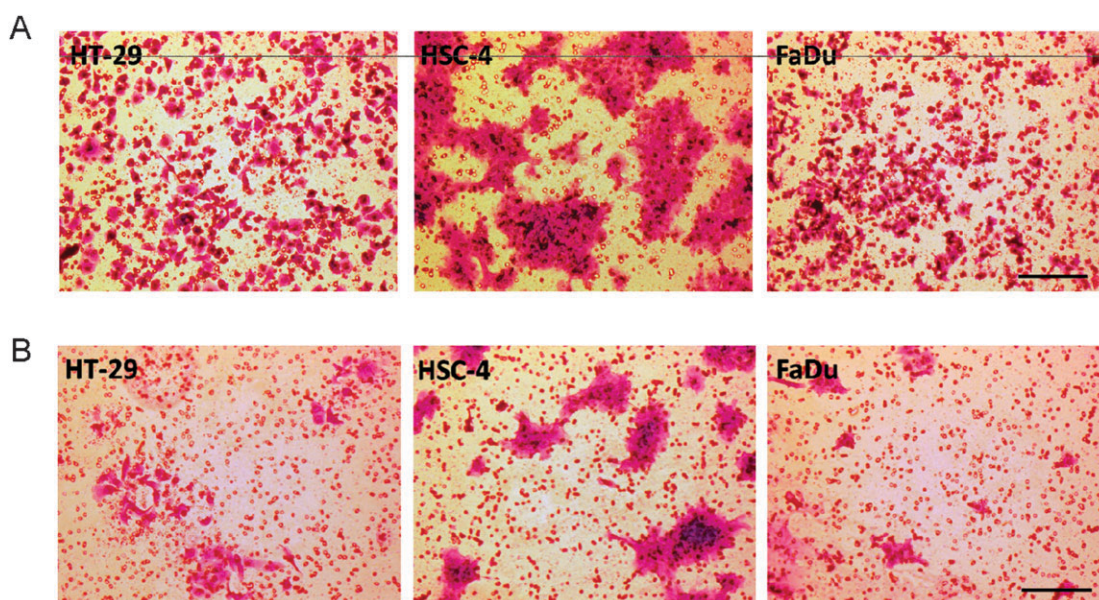


Fig. 2 Characteristic micrographs showing the migratory and invasive behaviour of the cancer cell lines HT-29, FaDu and HSC-4. Invasion and migration recordings were performed using Chemicon assays based on the well-established Boyden chamber. The migration or invasion of cells is determined by counting stained cells attached to the lower side of the porous polycarbonate filter after 15 h (migration) or 72 h (invasion). (A) Migration assay: microscopic field of view in which the number of migrated cells is determined, scale bar is 50 μm . (B) Invasion assay: microscopic field of view in which the number of invaded cells is determined, scale bar is 50 μm .

For studying the cells microscopically, cell inoculation was performed under the same conditions but on quartz resonators with thinner, semi-transparent electrodes. Cells could be monitored using an inverse phase contrast microscope (Olympus CKX 41, Hamburg, Germany). Cell-free resonators either immersed in culture medium or distilled water were used as controls to monitor noise of resonance parameters in the absence of cell adhesion or motility. Further control experiments comprise frequency fluctuations of resonators in contact to glycerol–water mixtures (60:40) as this solution imposes a similar acoustic load upon the resonator as living cells, however, with thermal/viscous noise as the only source for frequency variations.¹¹ Ultimately, all cellular activities were irreversibly stopped by chemical fixation using 0.5% glutaraldehyde (Sigma-Aldrich, Germany) to ensure that the observed frequency fluctuations originate from biological activity rather than changes of the load situation.

QCM setup and data acquisition

A quartz crystal microbalance consists of a thin piezoelectric resonator that oscillates in shear mode with an exceptional high quality (Q) factor allowing the deposition of a foreign mass to be sensed in sub-nanogram quantities. Besides rigid masses, the sensor is also responsive to changes in the viscoelasticity of the attached film. In aqueous solution the QCM displays a damped shear wave penetrating only a few hundred nanometres into the bulk solution. The penetration depth increases, however, with increasing viscosity. Among various alternatives based on either active or passive modes of QCM operation, we chose the dissipative QCM (D-QCM, frequently termed QCM-DTM), which monitors the free oscillation of a quartz resonator. The dissipative QCM was first introduced by

Kasemo and coworkers and has since found its way into many biomedical applications.^{42,43}

We use a home-made D-QCM setup (Fig. 1, based on Reiss *et al.*⁴⁴). The quartz resonator is excited to perform mechanical oscillations near its fundamental resonance of 5 MHz by a 80 MHz signal generator (Agilent 33250A, National Instruments). After a few microseconds of stable, forced oscillation the excitation source is disconnected from the quartz allowing a free but damped oscillation of the resonator. This procedure is repeated periodically. The free decay is read out by an A/D-converter (NI 5911, National Instruments) sampling at 100 MHz and the parameters of a damped sine wave transfer function ($U = U_0 e^{-t\delta} \sin(2\pi f t + \phi)$) are fitted to the experimental data. Continuous fitting of the free oscillation provides the resonance frequency f and decay constant of the oscillation δ as a function of time. δ is directly proportional to energy dissipation D :

$$D = \frac{\delta}{\pi f_0} = \frac{R}{2\pi f_0 L} = \frac{2\Gamma}{f_0} \quad (1)$$

In eqn (1), R denotes the motional resistance of the oscillation and Γ the half-band-half-width (HBH-width). R , δ , and Γ have in common that they are related indicators for the energy dissipation of the quartz oscillation.

Resonance frequency f_0 and the decay constant δ are measured in a time dependent manner. As a consequence, temporal fluctuations of these parameters could be recorded in the millisecond regime. While energy dissipation is low for rigid masses on the quartz surface due to direct coupling to the quartz resonator without acoustic losses, viscoelastic bodies like cells exhibit a significant dissipation. A home-made measuring chamber was used ensuring a minimally damped resonator (Fig. 1).

ECIS setup and data acquisition

Micromotion of the various cell lines was also monitored by a home-made ECIS setup consisting of a lock-in amplifier (SR830, SRS Inc., Sunnyvale, CA) with an internal oscillator, a multiplexer with analogue switches for automatic, consecutive addressing of individual wells on the electrode array, and a PC for experiment control and data storage. The ECIS electrode arrays (type 8W1E) purchased from Applied Biophysics (Troy, USA) consist of eight separate wells, each possessing one gold microelectrode of 250 μm diameter and a counter electrode that is roughly 1000 $\times$ larger in surface area. In our ECIS setup, a 1 V AC signal is applied to the system through a 1 M Ω series resistor. The in-phase and out-of-phase voltages across the electrodes are recorded at 4 kHz using a sampling rate of 1 Hz. The in-phase voltage, which is directly proportional to the real part of the complex impedance, was used for further analysis as it provides the most sensitive readout. The sensing voltage applied to the cells is of the order of a few mV and can be considered as being non-invasive.

Besides micromotion recordings at a fixed frequency, the barrier function of the adherent human cancer cell lines was monitored. The barrier function can be expressed by means of the so-called transepithelial electrical resistance (TER), being a direct measure for the ionic permeability of the cell-cell junctions. TER values were extracted from frequency-resolved

impedance readings (10–10⁴ Hz) and subsequent equivalent circuit modeling according to the procedures described by Wegener *et al.*⁴⁵

D-QCM and ECIS fluctuation analysis

The raw data obtained from either QCM (Δf and $\Delta\delta$) or ECIS ($\Delta U_{\text{in-phase}}$) was processed and analysed as follows (termed fluctuation or F-QCM): time course data is divided into data blocks of 500 points (with an overlap of 50% between single blocks for F-QCM). After subtracting a linear trend to remove long term drifts from the time series, these raw datasets are subjected to Fourier transformation in order to gain power spectral density (PSD). The linear trend is presumably due to thermal sensor drift or long-term processes associated with growth or death of cells. The slope m of the PSD was determined by linear least square fits in a low frequency regime (0.03 to 0.01 Hz for ECIS²³ or from 0.5 to 5 mHz for F-QCM, see Fig. 3). For time resolved presentations of the slopes $m_{\text{QCM}}^{\text{PSD}}$ or $m_{\text{ECIS}}^{\text{PSD}}$, the numerical values were smoothed by adjacent averaging over four successive data points. The smoothed slopes are plotted as a function of time assuming they are quasi-stationary within the given time interval. For the specific fluctuation levels after full cell monolayer was established, slope values from the confluent stages were pooled for each cell line and mean values derived from three independent

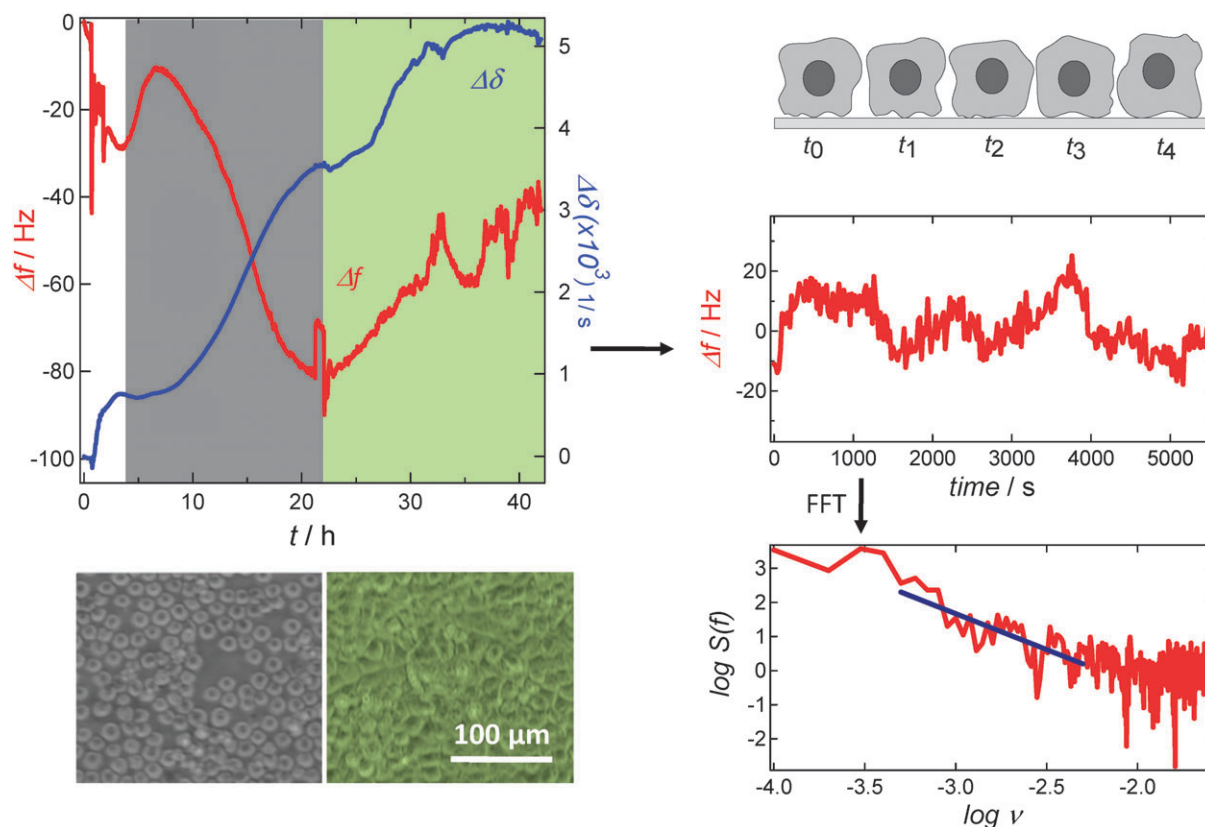


Fig. 3 Typical time course of Δf and $\Delta\delta$ as recorded in a D-QCM experiment after addition of 6.7×10^5 HT-29 cancer cells; the grey background denotes the time regime of attachment and spreading, whereas the green background highlights the cell monolayer in its confluent state. The labels Δf and $\Delta\delta$ denote the maximal shifts in frequency and dissipation. Fluctuations in frequency/dissipation can be extracted from fixed time intervals. Subsequent Fourier transform analysis provides the PSD (power spectral density). Slopes of the PSD curves in the frequency regime from 0.0005 to 0.005 Hz are used as one quantitative parameter to describe cell motility as are variance and detrended fluctuation analysis.

experiments are presented. The same procedure was carried out for variance analysis of Δf and $\Delta\delta$ fluctuations as well as $\Delta U_{\text{in-phase}}$. The variance was normalized to σ^2 of resonators/electrodes immersed in full culture medium as obtained from the same type of measurement (σ_{OCM}^2 or σ_{ECIS}^2) and are hence dimensionless. Finally, detrended fluctuation analysis (DFA)¹⁷ was applied to fluctuation data to identify and confirm cell-specific long-term correlations in the recorded noise and to validate power spectral data to ensure that indeed long-memory effects are observed. Long-memory dependence or persistence in time series and spatial data is generally associated with power-law correlations. In brief, the DFA comprises the following steps. First, the data sequence of length n is divided in n/l non-overlapping boxes. Within each box the local linear trend is removed from the original data and the variance about the detrended walk, which is defined as the difference between original data and the trend is averaged for each box of length l , denoted $F_d^2(l)$. Generally, $F_d^2 \propto l^\alpha$ holds, with α denoting the so called DFA exponent. While for uncorrelated noise $\alpha = 0.5$ is valid, long-range power-law correlation memory is characterized by $\alpha \neq 0.5$ ($\alpha > 1$). Note that $m = 2\alpha - 1$ holds, assuming that the power spectral density follows an inverse power law $\text{PSD} \propto f^m$ with $m < 0$.

Results

Spreading of cancer cells to other organs relies on cell motility and the invasion of adjacent connective tissue.^{4,46–48} Cell motility, widely recognized as an essential step in metastasis, is characterized by temporal changes in cell shape such as membrane ruffling, undulation, pseudopodal extensions leading to random or persistent walk of cells.^{49,50} An unusually increased motility found in cells that are otherwise in a rest stage is a sign of either tissue regeneration or neoplastic progression. Diagnostic tools that make use of this inherent connection between cell motility and cancer progression are sparse. The aim was here to scrutinize the potential of micromotion sensors based on QCM and ECIS as prognostic tools for invasiveness and migration ability of cancer cells. Hence, three cell lines of known malignancy FaDu, HT-29, and HSC-4 were employed as a test bed for micromotion assay based on fluctuation analysis. All cell lines are known to form confluent cell monolayers, an essential prerequisite for surface sensitive methods that are virtually blind to the third dimension. Confluent monolayers were chosen to detect minuscule changes in the vertical motility of otherwise connected cells indicative of a higher metastatic potential. Lateral migration of individual cells is not considered in this assay putting emphasis on the collective predominately vertical motion of 2D cell sheets. The spatially and temporally correlated movement of the complete confluent monolayer is expected to display characteristic dynamics of both the kinetics of cell adhesion bonds and reorganization of the cytoskeleton.

Boyden chamber migration and invasion assays

Classical Boyden chamber assays of migrative and invasive behavior of the three different cell lines were carried out as the gold standard for malignancy of cancer cells. Subsequently, resistance and frequency fluctuations as obtained from ECIS

Table 1 Mean migrated/invaded number of cells obtained from Boyden chamber migration and invasion assays for the three cancer cell lines HT-29, HSC-4 and FaDu ($n = 3$)

Cell line	Migration [cell no./FOV]	Invasion [cell no./FOV]
HT-29	400 ± 160	30 ± 8
HSC-4	204 ± 24	29 ± 8
FaDu	118 ± 30	12 ± 2

and F-QCM measurements, respectively, were compared with the established sequence of the metastatic potential.⁹ In a typical Boyden chamber assay the amount of cells per time migrating through an either uncoated or ECM-functionalized polycarbonate membrane is determined. At the end of the assay the cells are stained and counted. In Fig. 2A and B, optical micrographs from migration and invasion assays after staining of the cells are shown as an example. Table 1 summarizes the results of counting six areas of a defined 0.015 cm² field of view (FOV) per chamber after averaging for each cell line. The data comprises the mean and the standard deviation of three individual experiments. HT-29 colon cancer cells show the highest migration and invasion activity, followed closely by the HSC-4 cell line. HSC-4 cells form islands after migrating through the pores and exhibit a rather flattened morphology occupying almost twice the area as in its confluent stage. FaDu cells show little migratory and almost no invasive behaviour.

Analysis of F-QCM data

The following section outlines the principle approach to obtain fluctuation data from dissipative QCM of cells seeded on acoustic resonators. The general time course of cell growth measured by D-QCM is discussed and subdivided in different stages of cell layer development. Fig. 3 shows a typical D-QCM time course after seeding of $6\text{--}7 \times 10^5/\text{cm}^2$ synchronized HT-29 colon cancer cells. HT-29 cells are known to form confluent monolayers. After an initial frequency drop (dark grey area), which correspond to the process of spreading after initial attachment, the cells become confluent and express their morphological polarity (green area, 20–40 h after seeding). During this phase the monolayer displays increased frequency fluctuations. Time series data taken from this phase are further processed by stochastic analysis such as power spectral density estimation, variance analysis, and DFA as outlined above and an example is shown in Fig. 3. Cell growth monitoring by F-QCM was correlated with optical microscopy (Fig. 3, left/bottom).

D-QCM characterization of human cancer cells capable of forming confluent monolayers

Fig. 4 shows the frequency and dissipation response of the cells lines HT-29, FaDu, and HSC-4 upon seeding on the quartz resonator and the corresponding light micrographs. In order to focus our analysis on the micromechanical fluctuations of cells in a tissue-like environment, we seeded a sufficiently high number of cells into the QCM measurement chambers so that a confluent monolayer is immediately formed from the seeded cells. The arrows depict the time when confluency is reached.

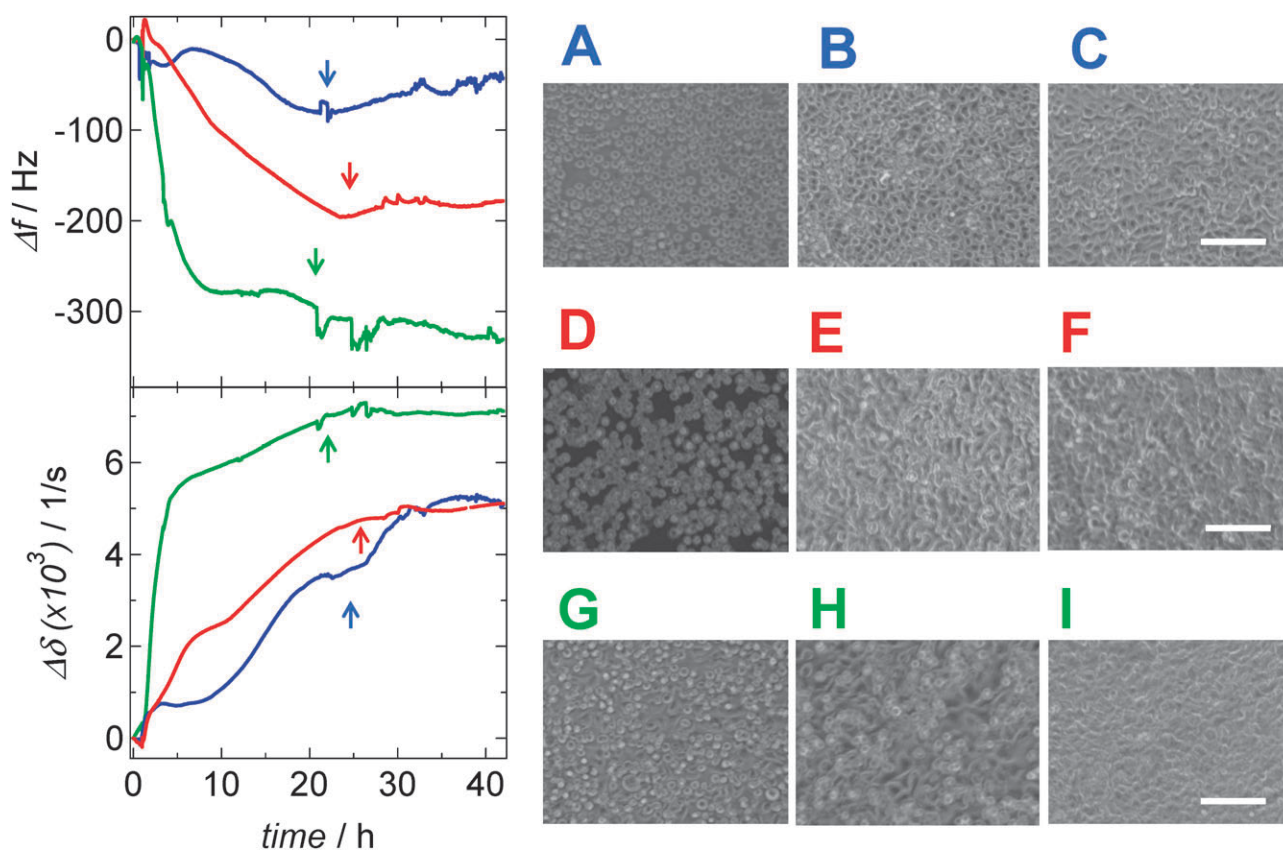


Fig. 4 Characteristic time courses of Δf and $\Delta\delta$ obtained from D-QCM experiments after addition of $6-7 \times 10^5$ cells that are capable of forming a confluent cell layer: colon carcinoma cell line HT-29 (●), hypopharyngeal squamous cancer line FaDu (●) and oral squamous epithelial carcinoma line HSC-4 (●). The highest acoustic load was produced by the HSC-4 cell line. Corresponding optical light micrographs are labelled in the same colour code shown on the right: (A–C) for HT-29 after 1 h, 16 h, 24 h; (D–F) for FaDu after 2 h, 24 h, 48 h; (G–I) for HSC-4 after 2 h, 4 h, 24 h, scale bar is 100 μm .

No multilayers are observed in the duration of the experiment, but a stationary state is found (see Fig. 4A–I). The mean values of Δf and $\Delta\delta$ obtained from this regime are summarized in Table 2. Additionally, the T_{ER} at confluency is reported to emphasize that cell–cell contacts have been formed. HSC-4 and FaDu show a generally stronger impact on the acoustic response of the resonator particularly on the frequency, while HT-29 cells mainly affect the dissipation. This reflects the difference in individual cell–substrate distance and natural variations of the micromechanical properties. Notably, FaDu cells often show a characteristic kink in the time course of Δf and $\Delta\delta$, usually between 6–8.5 h.

Remarkably, confluency of cells is accompanied by substantially increased frequency and dissipation fluctuations. This is most likely due to the continuous formation and decomposition of cell–substrate contacts of the cells in the

monolayer as well as fluctuations in the cells' height and viscoelasticity.

F-QCM and ECIS noise analysis of confluent cancer cells

Fluctuation analysis of confluent cell layers provide information about the general propensity to collective motility and as a consequence should display the malignancy of a cell line. Prior to fluctuation analysis of the various cancer cell lines grown onto quartz resonators, we confirmed that we are able to distinguish biologically induced fluctuations from other sources of noise by a series of control experiments. In order to rule out that the noise level in the presence of adherent cells is due to an increase in dissipative acoustic load imposed by the cell bodies, *i.e.* damping of the shear wave, we investigated the impact of a viscous glycerol–water mixture on the response of the shear oscillation and the noise in $\Delta f(t)$ as well as $\Delta\delta(t)$.

Table 2 Mean values of maximal frequency shift $-\Delta f$, damping parameter $\Delta\delta$, the ΔT_{ER} , and the time t needed to reach steady state upon loading the quartz plate with $6-7 \times 10^5$ cancer cells ($n = 3$)

Cell line	$-\Delta f \pm \text{SD}/\text{Hz}$	$\Delta\delta \pm \text{SD}/\text{s}^{-1}$	$\Delta T_{ER} \pm \text{SD}/\Omega \text{ cm}^2$	t/min
HSC-4	200 ± 100	5550 ± 160	170 ± 5	1800–2300
HT-29	110 ± 70	6410 ± 610	140 ± 15	1500–2000
FaDu	205 ± 35	5080 ± 110	80 ± 5	1000–1800

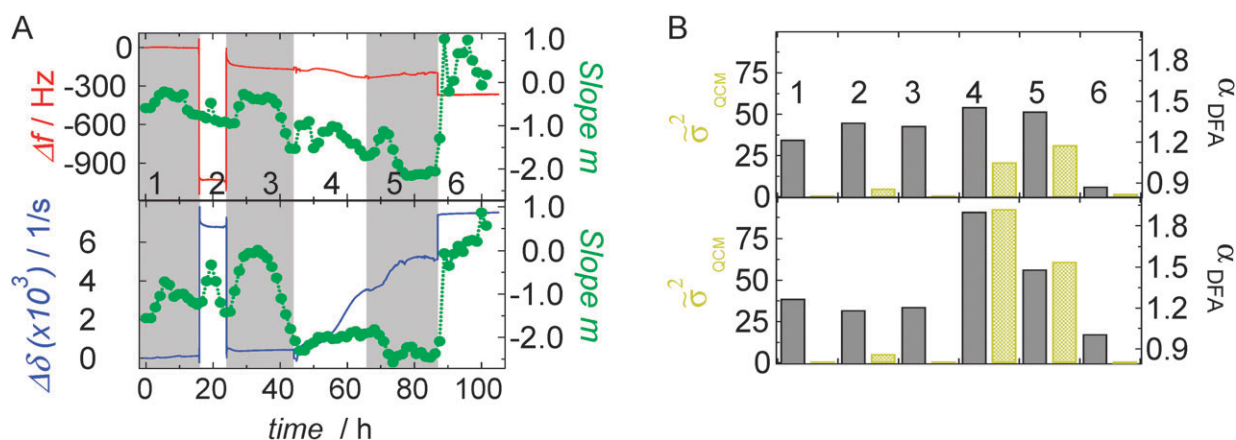


Fig. 5 Noise analysis of 5 MHz quartz resonator as a function of various acoustic loadings. 500 points from the original time series were used for each data point. (A) Δf (red), $\Delta\delta$ (blue) and the corresponding slopes $m_{\text{QCM}}^{\text{PSD}}(\Delta f, \Delta\delta)$ from the PSD (green) after addition of (1) de-ionised water, (2) a 60:40 (v/v) glycerol–water mixture, (3) culture medium followed by (4,5) addition of HT-29 cells. The experiment was finalized by (6) chemical fixation of the adherent cells after 50 h with 0.5% glutardialdehyde (GDA). (B) Variance analysis (σ_{QCM}^2 , yellow bar; size of data was 2000 points) obtained from the same regions as shown in (A).

Fig. 5A and B display a complex experiment, in which the quartz resonator was sequentially exposed to (1) de-ionised water, (2) a 60:40 (v/v) glycerol–water mixture, (3) culture medium followed by (4) a suspension of HT-29 cells and eventually (5) a confluent layer of HT-29. The experiment was finalized by (6) chemical fixation of the adherent cells after 50 h with 0.5% glutardialdehyde (GDA). The glycerol–water mixture provides the same viscous damping of the shear oscillation as measured for epithelial cells such as MDCK II.¹¹ Δf and $\Delta\delta$ of pure water was set to zero. As clearly visible, upon glycerol–water loading, Δf and $\Delta\delta$ reach new steady state values at -1000 Hz (Fig. 5A upper panel) and $+6700$ s⁻¹ (Fig. 5A lower panel), respectively. Fluctuation analysis only of the frequency time course shows a slight decrease in the $m_{\text{QCM}}^{\text{PSD}}(\Delta f)$ slope of the spectral density from -0.5 (water) to -0.7 (glycerol–water) accompanied by a slight increase (1 to 4) in normalized variance σ_{QCM}^2 both for frequency and damping. Accordingly, detrended fluctuation analysis (DFA) of frequency shows a slight increase in α from 1.22 to 1.34. Replacing the glycerol–water mixture with HEPES medium (phase 3) leads to recovery of Δf and $\Delta\delta$, however, as compared to pure water, to lower Δf and higher $\Delta\delta$ values, which we attribute to protein adsorption on the gold electrode as detailed previously.²⁴ Power spectral density estimation provides a decrease from $m_{\text{QCM}}^{\text{PSD}}(\Delta f) = -0.7$ (glycerol–water) to -0.45 (medium), and the values for σ_{QCM}^2 and α_{DFA} also recover to the parameters obtained for pure water. Upon addition of HT-29 cells, we can distinguish between the subconfluent (phase 4) and the confluent regime (phase 5). Attachment and spreading of the cells result in frequency shifts of -40 Hz with respect to pure medium. Fluctuation analysis of the frequency data from phase 4 provides a decrease of $m_{\text{QCM}}^{\text{PSD}}$ from -0.6 for the cell-free resonator to -1.27 for the cell-covered sensor, while σ_{QCM}^2 increases from 1 to 20 and α_{DFA} increases accordingly from 1.32 to 1.46. Analysis of fluctuations in the dissipation signal results in a similar trend with even more pronounced changes. Note, as we compute sliding averages of $m_{\text{QCM}}^{\text{PSD}}$, boundaries between the individual

phases are no longer clear cut. When a confluent monolayer is established on the surface (phase 5), maximum shifts for all fluctuation parameters are found with respect to cell-free resonators: increase in variance from 20 to 30 for frequency (or even beyond 50 for dissipation), $m_{\text{QCM}}^{\text{PSD}}$ of -2 and α_{DFA} values of > 1.5 . Here, the variance of the damping parameter shows an opposite trend due to strong runaway values in the subconfluent stage. GDA fixation of the cells increases the shift in Δf by additional -200 Hz, while $\Delta\delta$ increases by $+2250$ s⁻¹. Upon fixation, cell motility falls off, as expected and indicated by the readings of all fluctuation parameters (PSD slope, σ_{QCM}^2 , α_{DFA}). Although the fluctuations of the damping parameter provides larger slopes of the power density spectra, we restrict further analysis to frequency fluctuations due to higher robustness.

Fig. 6 summarizes the frequency response and fluctuation (PSD slope) as well as variance analysis of confluent cell lines HT-29, FaDu, and HSC-4. We found that the colon carcinoma line HT-29 shows the least impact on Δf , while the strongest change in the PSD slope and variance occurs upon confluency. Oppositely, FaDu cells display a substantially larger change in acoustic load accompanied by the smallest fluctuation amplitude and slope of the PSD. We attribute this to the stronger adhesion to the electrode and less motility. This is, however, not entirely causal. For instance, the oral squamous carcinoma line HSC-4 shows large fluctuation amplitudes and PSD slopes as well as considerable changes in resonance frequency and dissipation. Hence, strong adhesion does not necessarily imply slow dynamics. After 30 h of culture, pronounced frequency fluctuations can be found and optical micrographs indicate a fully established cell monolayer. It is noteworthy that fluctuation analysis provides two parameters, amplitude (variance) and frequency behaviour (PSD slope) that display two inherently different properties of the biological noise. For example, FaDu cells show an almost ten-fold increase in fluctuation amplitude, while the slope of the PSD, the long-time correlation, is altered negligibly. In summary, the order of noise intensities and correlation length

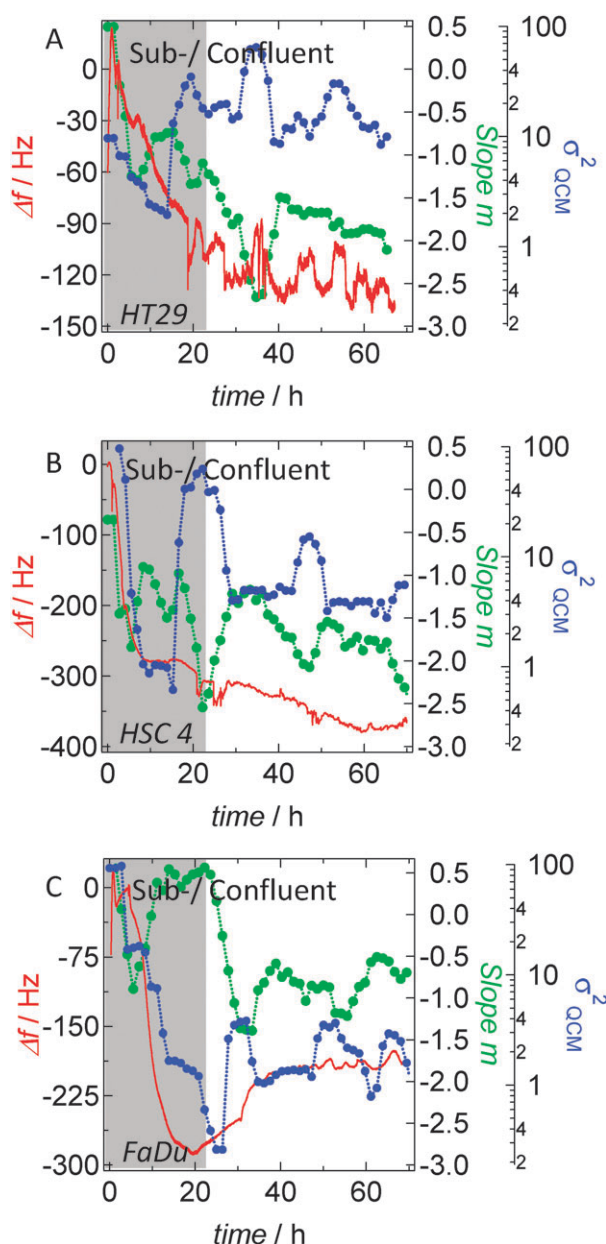


Fig. 6 Characteristic time courses of Δf and the corresponding slope m of the PSD-function ($m_{\text{QCM}}^{\text{PSD}}$) and variance (σ_{QCM}^2) for three human cancer cell lines ($6-7 \times 10^5$ cell inoculum) before/after a confluent monolayer is established: (A) HT-29; (B) HSC-4; (C) FaDu.

as extracted from the raw data by power spectral density estimation and variance analysis in decreasing order is: HT-29 > HSC-4 > FaDu. This sequence might correlate with decreasing cell-substrate dynamics within the three cell lines or alternatively a decrease of the micromechanical dynamics.

To distinguish between the viscoelastic fluctuations recorded by F-QCM and the fluctuation in the shape of the cells recorded by ECIS (Fig. 7), $m_{\text{ECIS}}^{\text{PSD}}$ and σ_{ECIS}^2 of all three confluent cancer cell lines were measured and analyzed over a period of 120 h. In Fig. 7 the time when the cells reached confluency is set to zero, only micromotion data resulting from confluent cell layers is considered. After 60 h, fixation with

0.5% GDA is carried out and the recorded fluctuations cease immediately. Micromotion as measured by ECIS shows the largest negative PSD slopes and variance for HSC-4, while F-QCM noise analysis reveals the largest slopes and variance for HT-29. FaDu cells display only intermediate σ_{ECIS}^2 and $m_{\text{ECIS}}^{\text{PSD}}$ in ECIS, while F-QCM noise analysis yields the smallest variance and smallest PSD slopes. Generally, we found: HSC-4 > FaDu > HT-29 for ECIS micromotion (PSD-slopes). However, F-QCM noise analysis especially by variance analysis discerns the individual motilities of the various cell lines more sensitively than ECIS-based micromotion recordings. Table 1, Table 3, and Fig. 8 summarize the results from migration, invasion, ECIS-micromotion and F-QCM viscoelastic fluctuations.

Discussion

The central aim of this study was to quantify the motility of human cancer cells by applying stochastic analysis algorithms to the fluctuations of the resonance frequencies of cell-covered shear wave resonators. It was the ultimate rationale behind this approach to correlate the cells' motility with their metastatic potential as it can be determined by classical Boyden chamber assays. For systems like adherent cells, the QCM readout provides, in principle, two parameters, the resonance frequency and the energy dissipation of the oscillation. Both of them were recorded and studied in terms of time series analysis. However, it turned out that the frequency-based time series is more robust and thus more suitable as the basis of fluctuation analysis. The fluctuating QCM signal can be considered as an integral cell parameter that does not reflect one particular cell property or structure but mirrors the motility of the cell as a whole or better, the collective and partly synchronized movement of several cells joined into small units.

Similar approaches that rely on the fluctuating pattern of the resonance frequency have been used for actively contracting cells in the past. With a low noise and fast acquiring network analyzer setup, Pax *et al.* were the first to analyze QCM fluctuations induced by actively contracting cardiomyocytes. The authors found periodic fluctuations in Δf and $\Delta \Gamma$ time courses due to the contractile activity of the cells with a frequency of 1.5 Hz.²⁰ In this present study, however, fluctuations of non-contractile cancer cells were recorded with shorter long-time correlation and significantly smaller fluctuation amplitudes compared to cardiomyocytes. As we have shown before,³⁸ metabolically driven cell fluctuations can be recorded by F-QCM approaches in a similar manner as it has been shown by impedimetric analysis of cell shape changes referred to as ECIS micromotion.^{17,21,51}

Previously, impedance spectroscopy (referred to as electric cell-substrate impedance sensing or short ECIS) has been used to discriminate between cancer cells that show an individually different metastatic potential.⁵² In that study, four breast cancer lines with increasing invasiveness showed different stationary impedance magnitudes and phase shifts accompanied by alterations in membrane capacitances. With respect to noise analysis, Lovelady *et al.*¹⁸ used power spectral density (PSD) analysis and detrended fluctuation analysis (DFA) of

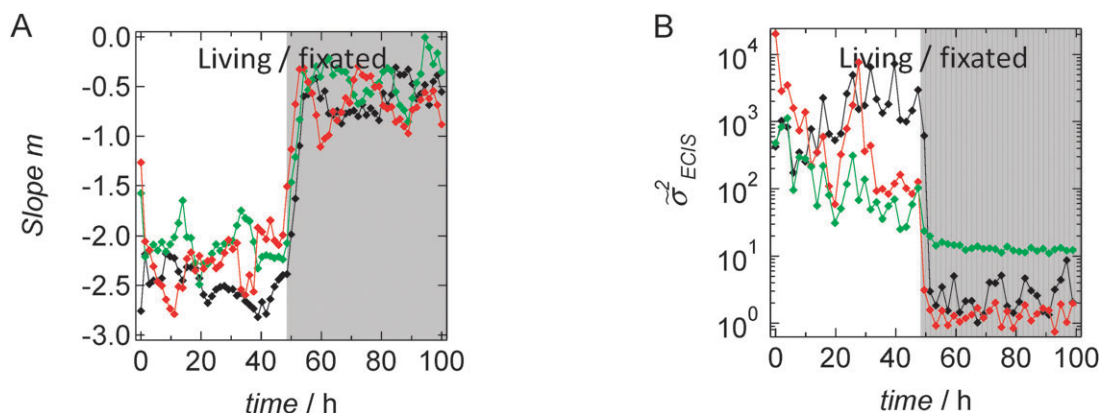


Fig. 7 Characteristic ECIS-micromotion (A) and variance recordings (B) for the three human cancer cell lines HT-29, FaDu and HSC-4. The time when a confluent cell monolayer is formed is set to zero; after 60 h, fixation with 0.5% GDA is carried out. Discrimination of cell lines in this assay is only related to shape fluctuations and not to viscoelastic changes as it might take place in D-QCM measurements.

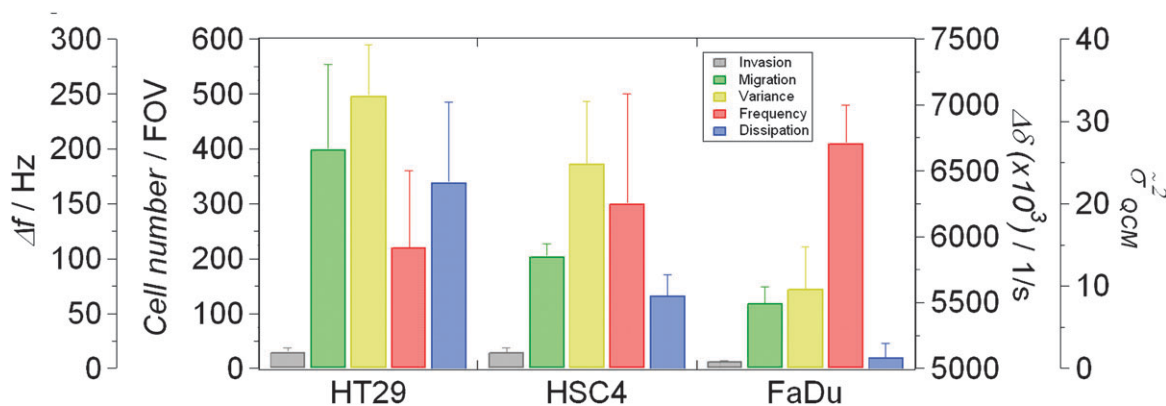


Fig. 8 Counted cells per field of view for migration (green) and invasion (grey) assay, error bars represent standard deviations ($n = 3$) vs. QCM parameters variance (σ^2 , yellow), frequency (red, in Hz) and dissipation (blue, in s^{-1}). Seeding density was adjusted to 210 000 cells in 300 μ l serum free medium.

Table 3 Mean F-QCM and ECIS Fourier transform slopes (m_{QCM}^{PSD} and m_{ECIS}^{PSD}) from least square linear fits in PSD, normalized variances ($\bar{\sigma}_{QCM}^2$ or $\bar{\sigma}_{ECIS}^2$) and DFA coefficients (α_{QCM}^{DFA} or α_{ECIS}^{DFA}) as well as mean cell numbers extracted from Boyden chamber migration and invasion assays for the three cancer cell lines HT-29, HSC-4 and FaDu. PSD slopes m_{QCM}^{PSD} and $\bar{\sigma}_{QCM}^2$ were computed from frequency fluctuations ($n = 3$)

Cell line	m_{QCM}^{PSD}	m_{ECIS}^{PSD}	$\bar{\sigma}_{QCM}^2$	$\bar{\sigma}_{ECIS}^2$	α_{QCM}^{DFA}	α_{ECIS}^{DFA}
HT-29	-1.8 ± 0.4	-2.1 ± 0.1	33.1 ± 6.2	167 ± 39	1.40 ± 0.04	1.51 ± 0.06
HSC-4	-1.5 ± 0.5	-2.5 ± 0.1	24.8 ± 7.6	1793 ± 4	1.37 ± 0.02	1.67 ± 0.02
FaDu	-0.6 ± 0.5	-2.2 ± 0.1	9.6 ± 5.2	1002 ± 29	1.22 ± 0.16	1.50 ± 0.05

the noise found in ECIS recordings with the goal to distinguish benign from malignant human ovarian surface epithelial cells. The results, however, were neither unique nor unambiguous. In a follow-up publication,⁵¹ cells with a higher fluctuation level (human umbilical vein endothelial cells, HUVECs) served as sensors for cytochalasin B cytotoxicity tests based on noise analysis.

Here, the question was addressed, whether fluctuation analysis of (i) micromechanical fluctuations monitored by acoustic shear wave resonators and (ii) cell shape fluctuations as measured by impedance analysis, are correlated with the metastatic potential of human cancer cells. As a test bed, three confluent cancer cell lines with a known malignant potency were employed.

In control experiments using highly viscous fluids and fixated cells, we could unequivocally show that fluctuations originating from living cells exceed all other sources of noise. Power spectral density generally reveals a power law dependence $\propto f^{-m}$ with $m \in [-0.6, -2.5]$ depending on the cell type. The value for m is indicative of long-memory stochastic processes and probably goes beyond a simple opening and closing of molecular bonds. Fractal behavior and long-time as well as long-range dependence have been observed in a numerous number of physical, geological, and biological processes. Time series and surface profiles have been characterized by their fractal dimension, a measure of roughness, and by the Hurst coefficient, a measure of long-memory dependence. The phenomena can be modeled and explained by stochastic

processes such as fractional Brownian motion, a directed version of the classical random walk.^{53–56} The assumption of statistical self-affinity implies a linear relationship between fractal dimension and Hurst coefficient and thereby links the two phenomena. The biological meaning of long-term correlations in cell shape fluctuations or volatile adhesion plaques lies in the correlation of individual stochastic adhesion bonds and the associated reorganization of the cytoskeleton. An increased long-term correlation or long-term memory implies that cell movement is less random in a classical sense but rather persistent and possibly cooperative. Our findings suggest that collective motion of cells occurs with higher amplitude for malignant cells but also with a higher temporal predictability. It is hence conceivable that a larger Hurst coefficient might be indicative of the onset of metastasis in an intact confluent monolayer due to a subtle but measurable change in the collective behavior of association and dissociation of non-covalent bonds triggered by a modified genetic program. The simplest way to look at enhanced time correlations is to consider spontaneous oscillations of the cell shape or cytoskeleton as they occur in nonlinear systems such as biological systems. The biological and physical origin of the fluctuations is most likely a complex interplay of changes in the cell–substrate contact and subtle shape/cell height variations. Random opening and closing of adhesion bonds (with τ , the lifetime of the bond) produce the frequency dependence of the PSD best described by $(\text{PSD} \propto \tau/(1 + (2\pi f\tau)^2))$ due to defined rate constants of association and dissociation.⁵⁷ The resulting f^{-2} tail would indeed explain—at least partly—the observed power laws. However, the area of the resonator is far too large to display fluctuations based on the individual opening and closing of bonds as pointed out by Lüthgens and Janshoff.⁵⁷ In fact, concerted movement of cell units comprising a few hundred to thousands of cells are responsible for the observed frequency changes. We know that around 500 000 cells produce a frequency shift of around 120 to 200 Hz. Since the frequency fluctuations cover around 1–2 Hz, it is conceivable that 10^3 cells participate in these movements considering the fact that the signal is mostly confined to the center of the resonator. Hence, we attribute the frequency fluctuations to collective motility rather than movement of single cells or even bonds.

The next question, which naturally arises is of what nature these collective fluctuations are. The QCM is an interface sensitive sensor that is capable of picking up small changes in the distance between the viscoelastic bodies and the resonator's surface. Height changes of adherent cells in the nanometre regime can also be measured due to the high sensitivity of the quartz resonator. Following the argument of Lucklum and coworkers, who relate changes in resonance frequency and dissipation to the viscoelastic properties of the attached material, a rough estimation is possible.⁵⁸ Assuming a storage modulus of adherent cells at 5 MHz of $G' = 10^5$ Pa and a loss modulus of $G'' = 10^6$ Pa·s a change in cell height from 4 μm to 4.1 μm translates to a shift of the resonance frequency of 2 Hz, which can be readily detected with our setup. In turn, changes in G' at constant cell height in the order of few kPa produce changes in f of about 1–3 Hz. Hence, it is difficult to determine whether shape fluctuations or

viscoelastic changes dominate the frequency noise. Power spectral density analysis of resistance fluctuations as a measure for micromotion in ECIS displays slopes that are generally larger ($m \in [-2.1, -3.0]$) compared to those found in F-QCM fluctuations (see Table 3). Hence, we assume that even though both methods are sensitive to cell shape changes, different information is retrieved by F-QCM noise analysis. Recalling that the shear wave produced by the quartz resonator decays exponentially in a viscous fluid it is straightforward to propose that the F-QCM is more sensitive towards changes in the cell–substrate contact regime compared to changes that occur in regions of the cell body that are more distant to the surface. Undoubtedly, metabolically driven motions of the cells can be monitored in a non-invasive fashion by both ECIS and F-QCM noise analysis, but the question is whether the fluctuations correlate with classical tests of invasiveness and migration behavior.

The cell motility measured by fluctuation analysis of the resonance frequency (PSD slope, variance, DFA) was found to decrease in the following order: HT-29 > HSC-4 >> FaDu. Fluctuation analysis of the resonance frequency was compared to ECIS micromotion and classical migration and invasion tests using a Boyden chamber. In general, we can only confirm the trend observed in the classical Boyden chamber assay with F-QCM-based noise analysis. We found that variance analysis of the resonance frequency provides the most sensitive measure for the metastatic potential while the PSD slopes undergo only slight changes (and present overlapping standard deviations). For instance, HT-29 and HSC-4 cells are characterized by the highest migration and invasion potential as confirmed by F-QCM-based variance analysis. Various publications on, for instance, efficiency of matrix–metalloprotease activity, draw a similar picture: the three human cancer cell lines studied here are considered to be gradually invasive. HT-29 are often used as a model system in experimental invasion studies, while FaDu and HSC-4 are reported to be slightly and moderately invasive, respectively.^{59–62}

Conclusions

A novel sensor approach based on resonance frequency fluctuations of F-QCM measurements is introduced to assay cell motility of human cancer cells. The method allows label-free and non-invasive monitoring of the cell body dynamics. Frequency fluctuations reproduce the metastatic potential that was determined by conventional assays based on the well-established Boyden chamber approach. Long-memory stochastic processes were found to govern the response of the adherent cells displayed in both F-QCM and ECIS micromotion measurements.

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