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Maximilian Oberleitner

Label-free and Multi-parametric Monitoring of Cell-based Assays with Substrate-embedded Sensors

Doctoral Thesis accepted by
the University of Regensburg, Germany

Springer

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Supervisor's Foreword

Throughout the last decades elaborate laboratory techniques to isolate and culture mammalian cells have been developed and continuously improved so that cell culture models from almost any mammalian tissue are available today for experiments *ex vivo*. This development has been originally motivated by the perspective to study one particular cell type apart from the complexity of an entire organism under well-defined laboratory conditions on a molecular scale. But cultured cells are not just simplified study objects to understand the molecular mechanisms of life, they also serve as valuable tools in bioanalysis when used as sensory elements in cell-based assays (CBAs). In CBAs the cells are exposed to a chemical, biological or physical challenge along a well-defined experimental protocol and the response of the cells to this challenge is used as a *biomarker*. When all experimental parameters are properly selected, CBAs provide a first and valuable estimate for the corresponding tissue response within the living organism. In this sense, CBAs are considered to be an intermediate between complex testing in living animals and simple, binary or ternary molecular assay systems. The number of applications for cell-based assays (CBAs) is huge and steadily increasing in all branches of biomedical research. For a successful assay it is indispensable but not sufficient to have an appropriate cell culture model available. It is equally important to have sensitive experimental strategies to *monitor* the behavior of these cells upon exposure to drugs, toxins, nanomaterials or other stressors. Moreover, the response of the cells to a given stimulus needs to be measured quantitatively in order to determine threshold concentrations, to establish structure-activity relationships or to compare different classes of compounds within one assay. Two different strategies have evolved to monitor and analyze cell-based assays. They are classified as *label-based* or *label-free* readout approaches dependent on whether they rely on chemical additives (fluorescent probes, antibodies, chromophores, etc.) to make the cell response measurable or not. Label-free approaches do not rely on chemical

detection principles but measure physical quantities (impedance, refractive index, viscoelasticity, etc.) to quantify the cell response (see also Sperber et al. 2016[1]).

Maximilian Oberleitner has devoted his thesis to the optimization, extension and combination of existing label-free approaches for monitoring cells in culture. The heart of his work is the *quartz crystal microbalance (QCM)* technique which has a long track record as a mass-sensitive tool to study molecular adsorption processes at the solid-liquid interface. In recent years it had been shown that these acoustic devices are also well-suited to report on the adhesion of cells, their viability and their cytomechanics. One of the weak spots of this technique has been its limited throughput. Max has tackled this problem by developing a scalable concept for multichannel QCM with more than one readout electrode per resonator and he applied it to monitor cell-based assays of various types. Another problem with QCM-based cell monitoring is the limited information content. Analysis of the resonant oscillation of QCM-sensors provides a maximum of two quantitative parameters, the resonance frequency and energy dissipation. Even though these are available as a function of time, the description of living systems asks for more independent information. It was Max's strategy to improve the device in this respect by combining it with other label-free readout approaches that provide an independent perspective of the cell response. And here he made efficient use of his 'multichannel QCM concept' developed before with coplanar electrodes on the surface of the quartz resonator. In addition to exciting the resonator's shear oscillation the surface electrodes were used to record electrochemical impedance spectra of the cells under study. This technique by itself has been known for many years and it is referred to as *electric cell-substrate impedance sensing*—or shortly ECIS. In combination with almost simultaneous QCM readings, the QCM-ECIS approach provides the viscoelastic and dielectric properties of the cells grown on the surface electrodes at the same time. Thus, the information content has been truly improved by another non-invasive readout approach. In this very same line Max has also combined QCM devices with optochemical sensing (OCS) of oxygen or temperature. The former was used to monitor the cells' respiration in parallel to recording their viscoelastic and dielectric properties. The latter was important to quantify the surface temperature when the quartz resonator is driven with elevated amplitudes in actuator applications. Both OCS-approaches were based on coating the resonator with polymer films that were doped with fluorescent indicators for oxygen or temperature. Thus, the title of this thesis indeed boils down its content: label-free, multi-parametric monitoring of cell-based assays with substrate-embedded sensors.

Max's scientific rigor in characterizing the performance of any new device in combination with his attention to detail make this thesis a highly informative reference for anybody working in the field of cell-based assays. No other publication

[1]M. Sperber, C. Hupf, M.-M. Lemberger, B. Goricnik, N. Hinterreiter, S. Lukic, M. Oberleitner, J. A. Stolwijk, and J. Wegener, "Monitoring the Impact of Nanomaterials on Animal Cells by Impedance Analysis: A Noninvasive, Label-Free, and Multimodal Approach", in *Measuring Biological Impacts of Nanomaterials* (Ed.: J. Wegener), Springer International Publishing, Cham, 2016, 45–108.

format than a thesis would allow spreading out all the experimental information that is relevant for a qualified judgement of different technical concepts. Most important to me is the enormous work that Max dedicated to the reproducibility of his approaches. Every assay was repeated as often as necessary to get reliable information about reproducibility and performance—always based on well-known statistical concepts. This last point may sound trivial. But looking through the scientific literature reveals that it is unfortunately not. Max's enthusiasm and talent to support concepts or experimental results by graphical elements provides a value by itself and will be appreciated by his readers. His dedication and determination paid off. All in all it has been a great pleasure to supervise this thesis and to work with Max in the lab.

Regensburg, Germany
August 2016

Prof. Dr. Joachim Wegener

Parts of this thesis have been published in the following journal articles

Related publications

1. M. Sperber, C. Hupf, M.-M. Lemberger, B. Goricnik, N. Hinterreiter, S. Lukic, **M. Oberleitner**, J.A. Stolwijk, and J. Wegener, "Monitoring the Impact of Nanomaterials on Animal Cells by Impedance Analysis: A Noninvasive, Label-Free, and Multimodal Approach", in *Measuring Biological Impacts of Nanomaterials* (Ed.: J. Wegener), Springer International Publishing, Cham, **2016**, 45–108.
2. K. Hajek, C. Schmittlein, **M. Oberleitner**, I. Shin, and J. Wegener, "Biosensors", in *eLS*, John Wiley & Sons, Ltd., Chichester, **2016**.

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Regensburg, Germany Maximilian Oberleitner
July 2016

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About the Author

Maximilian Oberleitner born 1983 in southern Bavaria/Germany, graduated from the University of Regensburg (Germany) with a Diploma in Chemistry in 2008. Based on this doctoral thesis "Label-free and Multi-parametric Monitoring of Cell-based Assays with Substrate-embedded Sensors" he obtained his Ph.D. in Analytical Chemistry in 2016 at the Institute of Analytical Chemistry, Chemo- and Biosensors at the University of Regensburg under the supervision of Prof. Dr. Joachim Wegener. His research work was focused on the development and improvement of substrate-integrated sensors for the analysis of living cells. These sensors enable the monitoring of cell-substrate interactions and the real-time analysis of assays with adherently grown cells in a non-invasive, label-free and—by combinations of the different types of sensors—even in a multi-parametric manner. Since 2015 he works at a global healthcare company in Austria. As project leader in the quality unit he is responsible for stability studies and quality evaluations of antibiotics.

Abbreviations and Acronyms

+ctrl	Positive Control
1ElQ	1-Electrode Quartz; 5 MHz AT-cut quartz disk ($Ø_q$ = 14 mm) with one electrode on either side ($Ø_E$ = 6 mm)
2,4-DNP	2, 4-Dinitrophenol
2-D	Two-dimensional
2ElQ	2-Electrode Quartz; 5 MHz AT-cut quartz disk ($Ø_q$ = 14 mm) with two electrodes on either side ($Ø_E$ = 3.5mm)
3-D	Three-dimensional
8-CPT-cAMP	8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate
8W1E™	ECIS array comprising 8 wells with 1 working electrode in each; trademark of Applied Biophysics, Troy, NY, USA
AC	Alternating Current
AFM	Atomic Force Microscopy
AJ	Adherens Junction
ATP	Adenosine Triphosphate
BAEC	Bovine Aortic Endothelial Cells
BCEC	Bovine Corneal Endothelial Cells
BOD	Biological Oxygen Demand
BPAEC	Bovine Pulmonary Artery Endothelial Cells
cAMP	Adenosine 3',5'-cyclic monophosphate
CBB	Cell-Based Biosensor/ Biosensing
cD	Cytochalasin D
CE	Counter Electrode
CMS®	Cell Monitoring System [64]
CPT-cAMP	*see* 8-CPT-cAMP
ctrl	Control
−ctrl	Negative Control
DAPI	4',6-diamidin-2-phenylindol

DC	Direct Current
DM	Dichroitic Mirror
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DO	Dissolved Oxygen
DSMZ	German Collection of Microorganisms and Cell Cultures
$EBSS^{--}$	Earles' Balanced Salt Solution, w/o Ca^{2+} and Mg^{2+}
$EBSS^{+-}$	Earles' Balanced Salt Solution, w/ Ca^{2+} and w/o Mg^{2+}
$EBSS^{-+}$	Earles' Balanced Salt Solution, w/o Ca^{2+} and w/ Mg^{2+}
$EBSS^{++}$	Earles' Balanced Salt Solution, w/ Ca^{2+} and Mg^{2+}
EC	Epithelial Cells
ECAR	Extracellular Acidification Rate
ECIS®	Electric Cell-Substrate Impedance Sensing; registered trademark of Applied Biophysics, Troy, NY, USA
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ELPO	Electroporation
EnFET	Enzyme Field-Effect Transistor
$Eu(benzac)_3(phen)$	Tris(benzoylacetonato)-mono(phenanthroline)-europium (III)
$Eu(dnm)_3(topo)_2$	Tris(dinaphthoylmethane)-bis(trioctylphosphine oxide)-europium(III)
FA	Focal Adhesion
FCS	Fetal Calf Serum
FED	Field-Effect Device
FITC	Fluorescein Isothiocyanate
FLIM	Fluorescence (Phosphorescence) Lifetime Imaging
FN	Fibronectin
GA	Glutaraldehyde
GJ	Gap Junction
GOx	Glucose Oxidase Enzyme
HAEC	Human Aortic Endothelial Cells
HC	Hydrocortisone
HCS	High-Content Screening
HD	Hemidesmosome
HTS	High-Throughput Screening
HUAEC	Human Umbilical Artery Endothelial Cells
HUVEC	Human Umbilical Vein Endothelial Cells
IA	Impedance Analyzer
IDEs	Interdigital Electrodes
IF	Intermediate Filaments
IR	Infrared Radiation
IS	Impedance Spectroscopy
ISFET	Ion-Selective Field-Effect Transistor
ITO	Indium Tin Oxide

IUPAC	International Union of Pure and Applied Chemistry
LAPS	Light-Addressable Potentiometric Sensor
LED	Light-Emitting Diode
MDCK-II	Madin Darby Canine Kidney cell line, strain II
ME	Microelectrode
MEM	Minimum Essential Medium Eagle
MISFET	Metal-Insulation-Semiconductor Field-Effect Transistor
MLAPS	Multiple Light-Addressable Potentiometric Sensor
MLC	Metal-Ligand Complex
MOSFET	Metal-Oxide-Semiconductor Field-Effect Transistor
NRK	Normal Rat Kidney cell line, strain 52E
OCR	Oxygen Consumption Rate
OCS	Optical Chemical Sensor/ Sensing
PBS^{--}	Phosphate Buffered Saline, w/o Ca^{2+} and Mg^{2+}
PBS^{+}	Phosphate Buffered Saline, w/a^{2+} and Mg^{2+}
PEBBLE	Probes Encapsulated By Biologically Localized Embedding
PEDOT	Poly(3, 4-ethylenedioxythiophene)
PFA	Paraformaldehyde
PhoP	Photopolymer
PKA	Protein kinase A
PMT	Photomultiplier Tube
PSP	Pressure-Sensitive Paint
PSS	Poly(styrenesulfonate)
PtTFPP	5,10,15,20-Tetrakis-(2,3,4,5,6-pentafluorophenyl)-porphyrin-platinum(II)
QCM	Quartz Crystal Microbalance
Q-factor	Quality Factor; cf. Eq. (63)
REVS	Rupture Event Scanning
RI	Refractive Index
RIfS	Reflectometric Interference Spectroscopy
RLD	Rapid Lifetime Determination
ROI	Region of Interest
rpm	Rounds Per Minute
RT	Room Temperature
RWG	Resonant Waveguide Grating
SDM	Standard Deviation of Mean
SEM	Standard Error of Mean
SFM	Serum-Free Medium
SI	Supplementary Information
SPR	Surface Plasmon Resonance
TER, TEER	Transepithelial/Transendothelial Electrical Resistance
TIR	Total Internal Reflection
TJ	Tight Junction
TSM	Thickness Shear Mode

TSP	Temperature-Sensitive Paint
UV	Ultraviolet Radiation
VIS	Visible Radiation
V_{rms}	Root-Mean-Square Voltage
w/	with
w/o	without
WE	Working Electrode
ZO	Zonula Occludens

Symbols

$\varnothing$	Diameter
α	Model Parameter, $\alpha = r_C \cdot \sqrt{\rho_{sub}/d}\ \left[\Omega^{1/2} \cdot \mathrm{cm}\right]$
δ	Decay Length
ε	Electric Permittivity
η	Viscosity
θ_i	Angle of Incident Light
λ	Wavelength
ν	Wave Frequency
ρ	Density; Specific Resistance
τ	Luminescence Lifetime
φ	Phase Angle, Phase Shift
ω	Radial Frequency, $\omega = 2\pi f$
Φ	Electric Potential [V]; Quantum Yield
ψ	Electrical Flux, $\psi = \iint_A \vec{D} \cdot d\vec{A}[\mathrm{A} \cdot \mathrm{s}]$
A	Area
B	Susceptance
$\overline{c_{66}}$	Piezoelectrically Stiffened Quartz Elastic Constant, $\overline{c_{66}} = c_{66} + {e_{26}}^2/\varepsilon_{22}$, for AT-cut Quartz: $\overline{c_{66}}$ = 2.947 10 $\times$ ^{10} kg/m^{-1}/s^{-2}
c_p	Specific Heat Capacity
C	Capacitance; Circumference
d	Distance
d_q	Quartz Thickness, d_q = 330 μm for f_s = 5 MHz
$\vec{D}$	Electric Displacement Field, $\vec{D} = \varepsilon_0 \varepsilon_r \vec{E}\ [\mathrm{A} \cdot \mathrm{s} \cdot \mathrm{m}^{-2}]$
e	Euler's Number, Base of the Natural Logarithm, $e \approx$ 2.71828
e_{26}	Piezoelectric Stress Constant; for AT-cut Quartz: e_{26} = 9.54 $\times$ 10^{-2} A·s·m^{-2}
$\vec{E}$	Electrical Field; $\vec{E}(\vec{r}) = -\nabla\Phi(\vec{r})$

E	Young's Modulus
f	Frequency
F	Fractional voltage drop across a cell layer [%], cf. Eq. (67)
$\vec{F}$	Force
$\vec{g}$	Gravitational Acceleration
G	Conductance
$\underline{G}$	Complex Shear Modulus
h	Height; Plank Constant
$\underline{i}$	Complex Current
$\hat{\imath}$	Current Amplitude
I	Electrical Current; Luminescence Intensity
$\mathrm{Im}(\underline{Z})$	Imaginary Part of the Complex Impedance
j	Imaginary Unit
k	Spring Constant; Rate Constant
K_{SV}	Stern-Volmer Quenching Constant
L	Inductance
m	Mass
M	Molar Mass
n_i	Refractive Index of Medium i
N	Number of Values Used for Averaging
$p\mathrm{O}_2$	Oxygen Partial Pressure
p_{tot}	Total Pressure
q	Charge
Q	Quality Factor, Q-factor, $Q = 1/D = X_{tot}/R_{tot}$; Quencher
r	Radius; Damping Constant ($r = \eta_q \pi^2 / d_q^2$)
R	Resistance
$Re(\underline{Z})$	Real Part of the Complex Impedance
t	Time
T	Temperature
$\underline{u}$	Complex Voltage
$\hat{u}$	Voltage Amplitude
U	Voltage
v	Velocity
V	Volume
w	Gaussian Distribution Coefficient, $w^{air} = 2.84$, $w^{water} = 2.03$
x	Shear Amplitude; Displacement
X	Reactance
$\underline{Z}$	Complex Impedance
$\lvert Z \rvert$	Impedance Magnitude
Z	Impedance

Special (Bio)Chemical Reagents

2, 4-Dinitrophenol (2,4-DNP)	Sigma-Aldrich; St. Louis, MO, USA
4', 6-diamidin-2-phenylindol (DAPI)	Sigma-Aldrich; St. Louis, MO, USA
5, 10, 15, 20-Tetrakis-(2,3,4,5,6-pentafluorophenyl)-porphyrin-platinum(II) (PtTFPP)	Porphyrine Systems GbR; Appen, Germany
8-(4-Chlorophenylthio) adenosine 3',5'-cyclic monophosphate sodium salt (8-CPT-cAMP)	Sigma-Aldrich; St. Louis, MO, USA
Alexa Fluor® 488 phalloidin	Life Technologies; Carlsbad, CA, USA
Alexa Fluor® 546 rabbit anti-mouse IgG (H+L)	Life Technologies; Carlsbad, CA, USA
Cytochalasin D (cD)	Sigma-Aldrich; St. Louis, MO, USA
Dimethylsulfoxide (DMSO)	Sigma-Aldrich; St. Louis, MO, USA
FITC-Dextran 250 kDa	Sigma-Aldrich; St. Louis, MO, USA
Glutaraldehyde (GA)	Merck KGaA; Darmstadt, Germany
LIVE/DEAD® viability/ cytotoxicity kit	Molecular Probes, Life Technologies; Carlsbad, CA, USA

Paraformaldehyde (PFA)	Merck Schuchardt OHG; Hohenbrunn
Photoresist AZ® ECI 3027	Microchemicals; Ulm, Germany
Poly (vinylidene-chloride/ acrylonitrile) (80:20)	Polysciences, Inc.; Warrington, PA, USA
Sylgard® 182 silicone elastomer kit	Dow Corning; Midland, MI, USA
THF	Sigma-Aldrich; St. Louis, MO, USA
TI Prime	Microchemicals; Ulm, Germany
Toluene	Merck; Darmstadt, Germany
Tris(benzoylacetonato)-mono(phenanthroline)-europium (III) ($Eu(benzac)_3(phen)$)	Sigma-Aldrich; St. Louis, MO, USA
Tris(dinaphthoylmethane)-bis(trioctylphosphine oxide)-europium(III) ($Eu(dnm)_3(topo)_2$)	Synthesized at the institute, according to Peng's procedure [329]
TRITC-phalloidin	Sigma-Aldrich; St. Louis, MO, USA
Triton-X-100	Sigma-Aldrich; St. Louis, MO, USA
ZO-1 mouse monoclonal antibody	Life Technologies; Carlsbad, CA, USA