



Investigations of ring nanoelectrodes integrated into microwell arrays for the analysis of isolated mitochondria at the microscale

F. Sékli Belaïdi, G. Lemercier, V.S.R. Vajrala, D. Zigah, J. Launay, <u>P. Temple-Boyer</u>, S. Arbault



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Background

"Cancer, above all other diseases, has countless secondary causes. But, even for cancer, there is only one prime cause. Summarized in a few words, the prime cause of cancer is the replacement of the respiration of oxygen in normal body cells by a fermentation of sugar."

Otto Heinrich Warburg (1923)

M.G. Van der Heiden et al., Science (2009), Harvard university, USA



The Warburg effect:

from oxidative phosphorylation to aerobic glycolysis in cancer cell mitochondria

The metabolic pathways of glucose in normal cells

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Question, hypothesis and objective

Questions: How mitochondrial energetic metabolisms influence cancer cell proliferation and/or resistance to chemotherapy ?

Hypothesis: cells responsible for cancer proliferation and/or relapse have specific mitochondria-driven energetic and metabolic features...

Objective: Towards the analysis of mitochondrial metabolisms...



Development of nanohole arrays for trapping single mitochondria

S. Kumar et al., Anal. Chem (2015), University of Minnesota, USA



> Integration of nanohole arrays on Si_3N_4 -suspended membranes

- $\checkmark~$ Nanohole diameter and thickness: ~0.3 μm and ~0.3 μm
- Nanohole "trapping volume": ~100 aL

Application

- ✓ Trapping of single mitochondria into nanoholes using evaporation phenomena
- ✓ Study of mitochondrial metabolisms using fluorescence analysis

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Study of mitochondrial metabolisms



- The cell-mitochondria symbiosis
 - ✓ "Powerhouse of cell": consumption of pyruvate and oxygen O₂ for the ATP synthesis from ADP
 - ✓ Cell apoptosis through the production of ROS: H_2O_2 , O_2° ,...

Electrochemistry: development of ultra-microelectrodes (UME) for isolated mitochondria analysis...

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UME for single cell analysis (1)



Use of capillary-based ultra-micro-electrode (UME)

Characteristics

- Time-consuming, low yield and low reproducibility fabrication
- ✓ Complex experimental procedure
- No statistical analysis to overcome cell variability
- ✓ Partial detection of any exocytotic event (collection ratio ≤ 0.5)

Realization of planar UME using "Silicon" technology

Characteristics

- Mass fabrication with high yield and high reproducibility
- Easier experimental procedure: positioning cells on UME arrays...
- ✓ Multi-analysis in cell cultures
- ✓ Partial detection of any exocytotic event (collection ratio ≤ 0.5)

UME for single cell analysis: <u>a new solution...</u>



- Realization of recessed ring nanoelectrodes (RNE) into integrated microwells using "Silicon" technology
- Characteristics
 - ✓ Mass fabrication with high yield and high reproducibility
 - ✓ Experimental procedure: locating cells (mitochondria...) into microwell arrays !!!
 - Statistical multi-analysis in cell cultures
 - ✓ Improved detection of any exocytotic event with collection ratio approaching 1...

Development of recessed ring micro/nano-electrodes





C. Ma et al., ACS Nano (2013), Notre Dame University, USA

Integration of "ring-type" micro/nano-electrodes into microwells

- ✓ Electrode surface: ~30 μ m² / ~ 0.2 μ m²
- $\checkmark~$ Microwell diameters and depths: ~50 μm and ~8 μm / ~0.5 μm and ~1 μm
- ✓ Microwell volumes: ~16 pL / ~250 aL
- ✓ Applications: immunodetection / electrochemical investigations at the nanoscale
- ✓ Not adapted to mitochondria analysis up to now (requested volumes: ~0.5 pL)

<u>COMSOL™ simulation</u> for an optimal structure design

- 3D-axysymetric modelling of diffusion phenomena into the microwell
- Optimization of the collection ratio





✓ ϕ_{out} : flow of species collected on the ring nanoelectrode (RNE)

Goals

✓ Definition of microwell and RNE geometrical parameters in the frame of the "single mitochondrion" analysis (typical scale: ~1 µm)



Optimization of the collection ratio



Microwell geometry

Increase of the microwell aspect ratio H/R

Ring nanoelectrode characteristics

- Increase of the thickness E
- ✓ Location at the microwell mid-depth

Collection ratio values: from 0.75 to 0.95

✓ Towards full detection of any exocytotic event



Towards single mitochondria analysis in the frame of "Silicon" technologies





- Use of transparent substrate: compatibility with optical analysis
- > Fabrication of a SiO₂-based microwell

\checkmark	Height: H ≈ 5.2 μm	microwell
\checkmark	Radius R: 3 and 4.5 µm	volume: ~ 0.3 pL

- > Integration of a platinum-based, recessed, ring nanoelectrode
 - ✓ Thickness: $E \approx 0.2 \ \mu m$
 - ✓ Recess depth: $H_2 \approx 2.5 \ \mu m$

Fabrication process

F. Sékli Belaïdi et al., S&A: B (2016), Toulouse University, France



Step 0 (optional): deposition and patterning of a Ti/Pt/Ti layer on a B33 glass substrate



<u>Step 1:</u> deposition of a SiO₂/Ti/Pt/Ti/SiO₂ stacking



Step 3: opening contacts in the upper SiO₂ layer



<u>Step 2 (critical):</u> reactive ion etching of the SiO₂/Ti/Pt/Ti/SiO₂ stacking

The ElecWell concept



Integration of recessed ring nanoelectrodes (RNE) into microwell arrays with bottom disk microelectrodes (DME): Application for electrochemical investigations...



...or without: application for mitochondria analysis

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Technological interference...



> Overetch of the upper SiO₂ layer during the lower layer etch

- ✓ Overetched length: L ≈ 0.3 μ m
- ✓ Fabrication of a platinum-based step

COMSOL simulation: slight collection ratio increase (+ 6-9%)

- ✓ Improvement associated to the ring nanoelectrode surface increase
- Final integration of recessed ring nanoelectrodes (RNE) (area: ~ 10 or 15 μm²) into microwell arrays

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<u>Technological achievements:</u> <u>the ElecWell platform</u>













Electrochemical characterization of a single RNE nanodevice (R = 4.5 μ m) in sulfuric acid (H₂SO₄ 0.5 M)



Cyclic voltammogram specific of platinum (use of a platinum counter electrode and a silver wire as pseudo-reference electrode)

<u>Electrochemical characterization of</u> <u>RNE arrays (Fc(di-MeOH) – 1 mM)</u>



X-axis: Potential (V vs Ag)

Definition of limit currents for individual RNE and associated RNE arrays (respect of proportionality)

✓ Agreement with UME electrochemical behaviour: hemispherical diffusion

✓ No diffusion phenomena overlapping from one microwell to another

<u>Theoretic study of</u> single recessed ring nanoelectrodes

\succ Calculation of limit currents I_{lim} thanks to:

- ✓ Recessed ultra-microelectrode theory
- ✓ Ring microelectrode model (A. Szabo, J. Phys. Chem. (1987), Maryland, USA)



Radius	Theoretical model	COMSOL™ Simulation	Experiment
3 µm	380 pA	390 pA	400 pA
4.5 µm	660 pA	620 pA	650 pA

Good agreement between theoretical, simulated and experimental results

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<u>RNE – DME system in generation-</u> collection mode (Fc(di-MeOH) – 1 mM)



- > Chronoamperometric experiments
 - ✓ E = 0.4 V vs Ag: ferrocene Fc oxidation on the bottom disk microelectrode
 ✓ E = 0.2 V vs Ag: ferrocenium Fc+ reduction on the recessed ring nanoelectrode
- > Experimental study of the collection ratio for R = 4.5 μ m

✓ $r_{\text{collection}} \approx 0.76$ (agreement with simulation: r = 0.80)

Application of the ElecWell-V1 platform for mitochondrial metabolism analysis





Experimental protocol

- ✓ Use of yeast mitochondria solution
- Flow system washing to prevent floating mitochondria in solution

Optical analysis using NADH fluorescence

- \checkmark ~ 20% of microwells filled by a single mitochondrion
- ✓ Use of 1000 x 1000 RNE arrays: parallel and simultaneous analysis of 200 000 mitochondria into microwells approximately...

Application of the ElecWell-V1 platform for mitochondrial metabolism analysis



Cyclic voltammetry (-0.85 / -0.08 V, scan rate: 4 V/s, 20s gap, Pt counter electrode, "Ag/AgCl wire" reference electrode)

- ✓ Reduction current at -0.7 V: O₂ detection
- Towards mitochondrial respiration monitoring...

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Application of the ElecWell-V1 platform for mitochondrial respiration analysis



> Monitoring mitochondrial respiration (~ 200 000 mitochondria)

- \checkmark Addition of ethanol: respiration starting and O₂ concentration decrease
- ✓ Addition of ADP (ATP synthesis): respiration "two-fold" enhancement
- ✓ Addition of antimycin A: respiration inhibition and O_2 concentration stabilization

Application of the ElecWell-V2 platform for mitochondrial metabolism analysis



- ➤ Use of 100 x 100 RNE arrays
- Similar results for the electrochemical monitoring of respiration phenomena for 2000 mitochondria into microwells...

Result synthesis

tested device	number of mitochondria	O ₂ consumption rate in EtOH	O ₂ consumption rate in EtOH + ADP
1000 x 1000 RNE array	200 000	0.2 pA/s per mitochondrion	0.38 pA/s per mitochondrion
100 x 100 RNE array	2 000	0.75 pA/s per mitochondrion	1.6 pA/s per mitochondrion

Estimation of mitochondrial oxygen consumption rate

- ✓ Respiration "two-fold" enhancement with and without ADP
- Ratio 4 between results obtained for the two different RNE-array devices...
- Presence of interfering mitochondria on the wafer surface or in solution...
 - ✓ Experimental protocol improvement: mitochondria preparation, functionalization process, fluid handling,...



Monitoring of respiration phenomena for ~ 10 000 mitochondria

Conclusion

- Integration of recessed ring nanoelectrodes (RNE) into microwell arrays for isolated mitochondria analysis
 - \checkmark Simulation and optimization
 - ✓ design and technological realizations on transparent glass substrate (compatibility with optical analysis)
 - ✓ Electrochemical investigations
 - ✓ First mitochondria analysis

Results

- Electrochemical behaviour of individual recessed RNE and recessed RNE arrays: agreement between theory, simulation and experiment
- Location of mitochondria into microwell arrays: filling ratio around 20%
- ✓ Monitoring the respiration of 10 000 mitochondria thanks to O₂ real-time analysis at the microscale...







Prospects

Towards the ElecWell-V3 platform...

- ✓ Realization of electrochemical nanocells...
- ✓ Towards 3 µm diameter microwell...
- ✓ Integration into microfluidic systems...

> From O_2 to H_2O_2 and ROS/RNS detection

- Development of black-platinum (BI-Pt) electrodeposition on recessed RNE nanodevices
- Improving the immobilization of mitochondria in microwell arrays
 - Preventing mitochondrial adhesion on the SiO₂ upper surface using PLL-PEG functionalization process

Towards mitochondrial metabolism analysis

- ✓ From multi to single mitochondria analysis
- \checkmark Comparison of mitochondria from safe and cancer cells
- ✓ Study of Warburg effect and other cancer-related phenomena









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