High-Content Toxicology Screening Using Massively Parallel, Multi-Phasic Cellular Biological Activity Detectors MP²-CBAD

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Edgewood Chemical and Biological Center (SBCCOM / ECBC)

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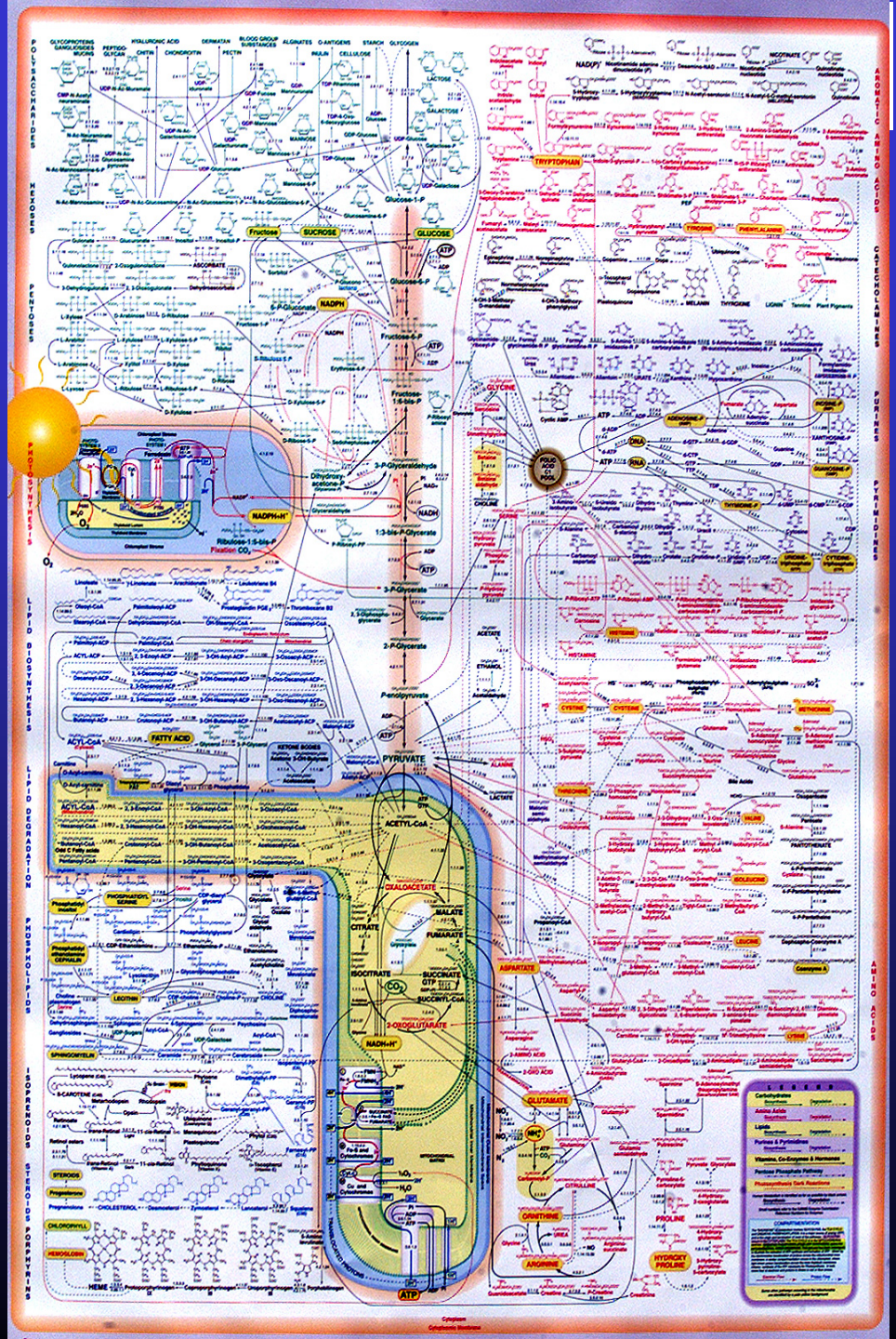
Objective

- Develop cell-based, fast-response metabolic sensing arrays for detection and discrimination of toxins or for use in drug screening efforts.
- Use massively parallel arrays of devices with multiple sensors and cell lines, subnanoliter volumes, and active microfluidics for ***rapid response and closed loop control of the extracellular space!***

- Massively Parallel, Multi-Phasic Cellular Biological Activity Detector (MP^2-CBAD)

VIBRE

Cell physiology is a giant linked network of metabolic and signaling pathways



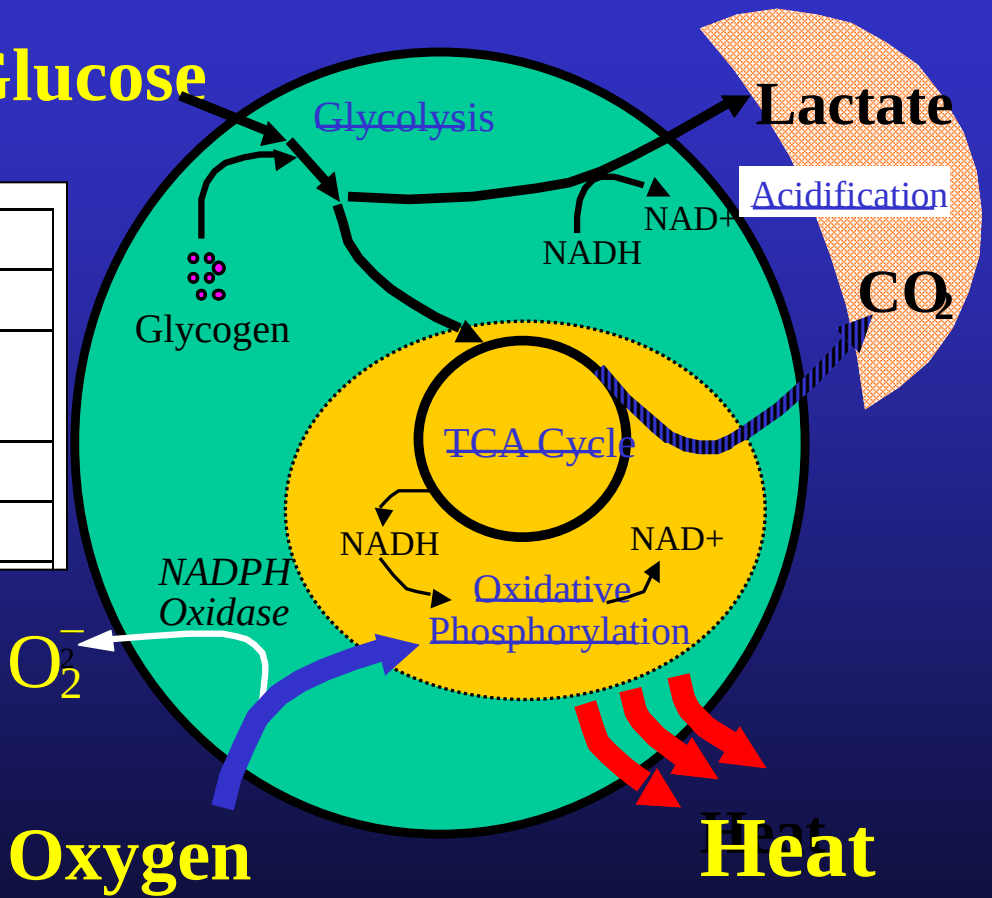
Cell-Based Biosensor as Generalized Toxicity Sensor

- We do not measure the toxin itself. We are measuring the impact of the toxin on cell physiology by probing cell functions!
  - **Metabolic pathways**
  - Signaling pathways
  - Electrical excitability
  - Cell-to-cell communication ……..

→ Intrinsic amplification

Simplified Metabolic Network

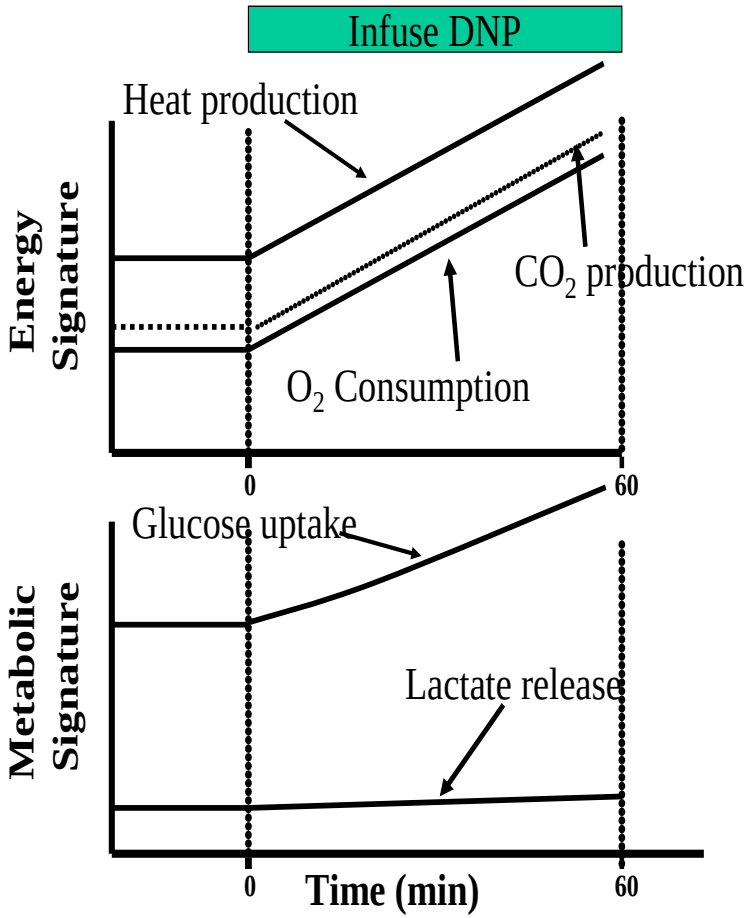
Glucose + 2 ADP + **2 NAD$^+$**	→	**2 Pyruvate** + 2 ATP + **2 NADH**
Pyruvate + **NADH**	→	**Lactate** + **NAD$^+$**
Pyruvate + CoA + **FAD** + GDP + **3 NAD$^+$** + **NAD(P)$^+$**	→	**3 CO_2** + **FADH2** + GTP + **3 NADH** + **NAD(P)H**
0.5 O_2 + 3 ADP + **NADH**	→	3 ATP + **NAD$^+$**
0.5 O_2 + 2 ADP + **$FADH_2$**	→	2 ATP + **FAD**

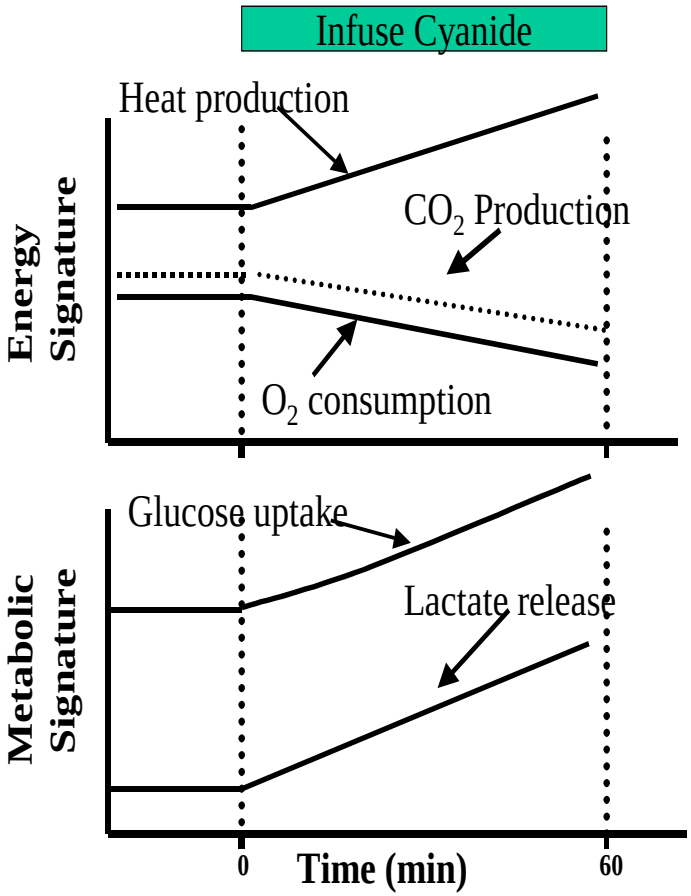


MP²-CBAD Discrimination

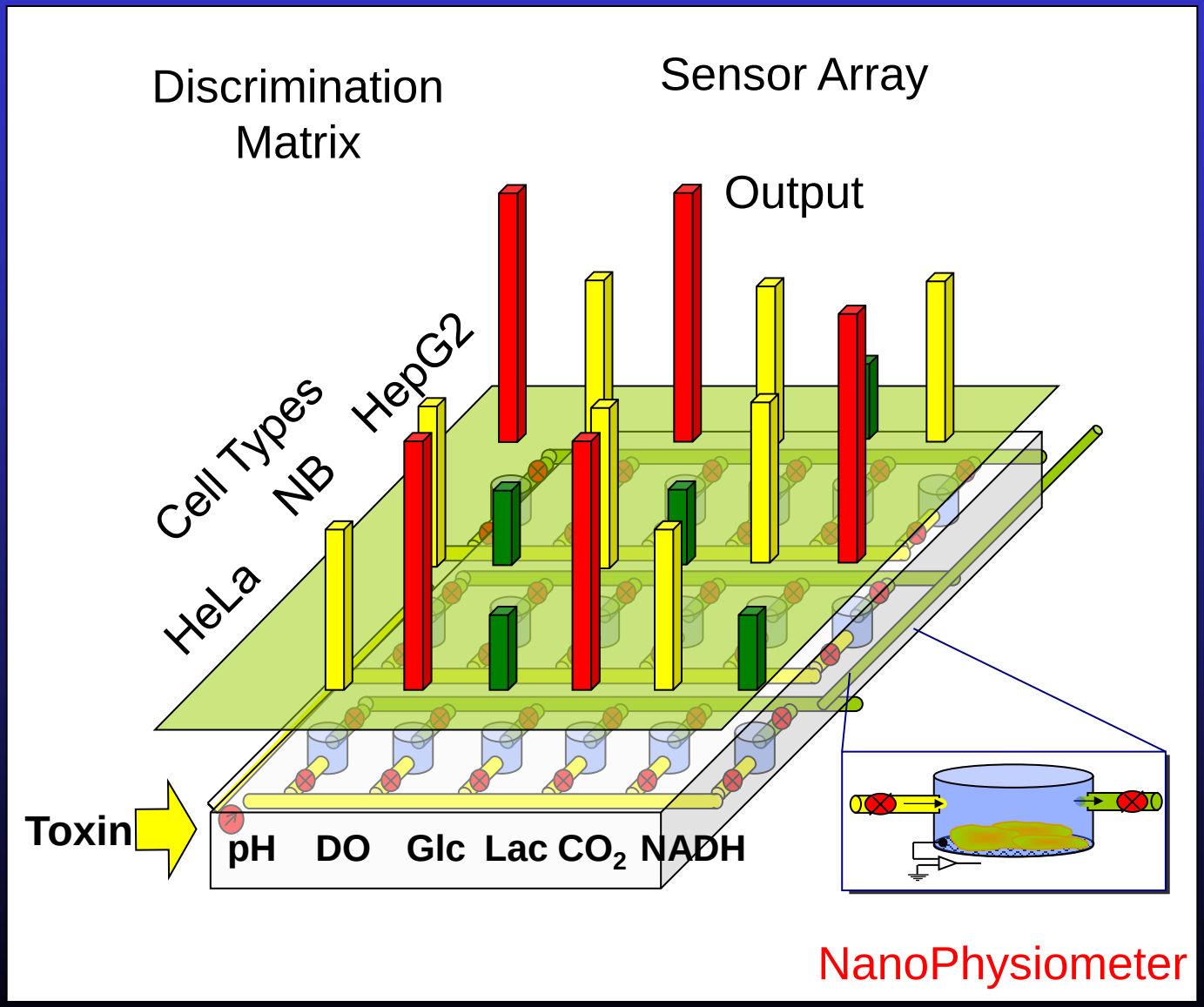
- Simultaneous monitoring of *multiple metabolic signals*
- Characteristic response in a *conditioned* environment
- Characteristic responses of cellular *phenotypes* to toxins
- Characteristic reaction *kinetics* of metabolic pathways

Discrimination: Simultaneous monitoring of multiple metabolic signals





MP²-CBAD Discrimination



Three Spatial/Temporal Scales for Cell-Line Screening and BioSignature Generation

- Milliliter – minutes to an hour

  96 and 24-well-plate cell culture
- Microliter – 10-100 seconds

  Modified Cytosensor MicroPhysiometer
- SubNanoliter – 10-100 milliseconds

  Vanderbilt NanoPhysiometer

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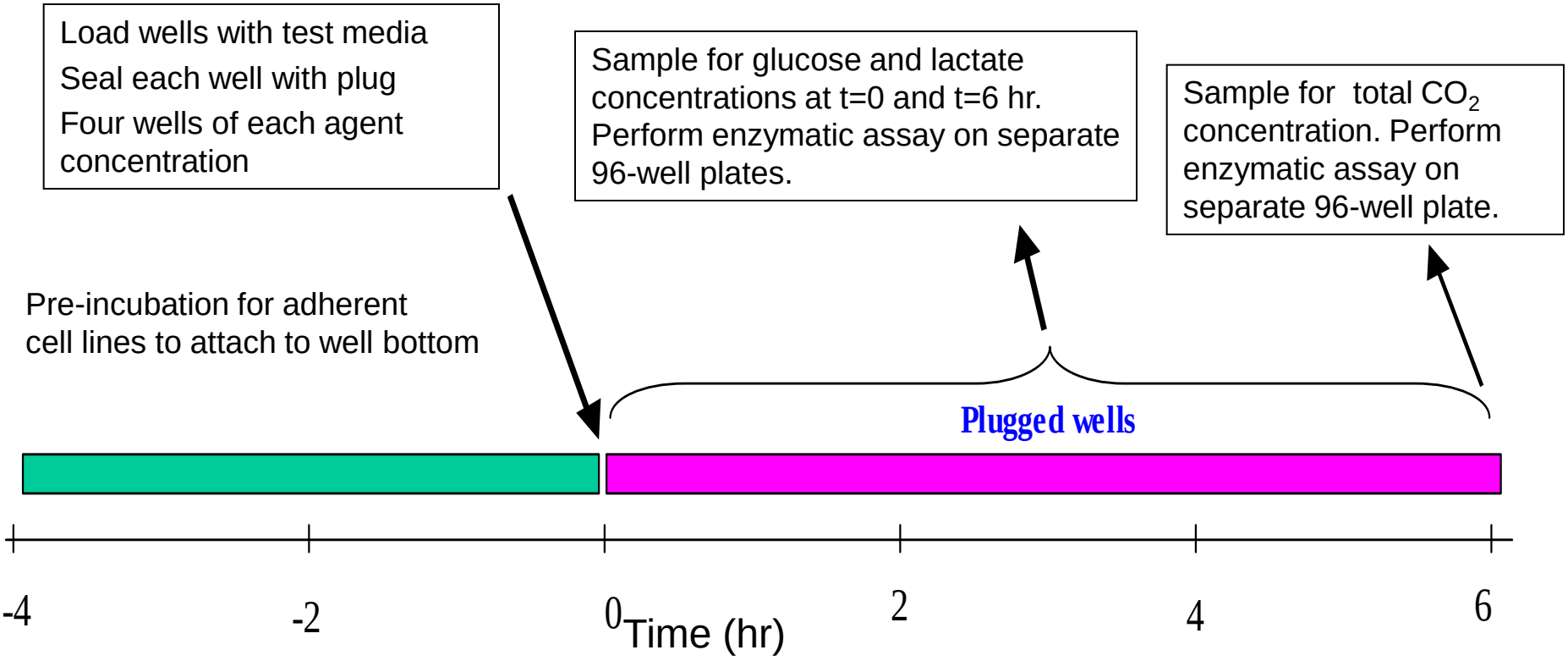
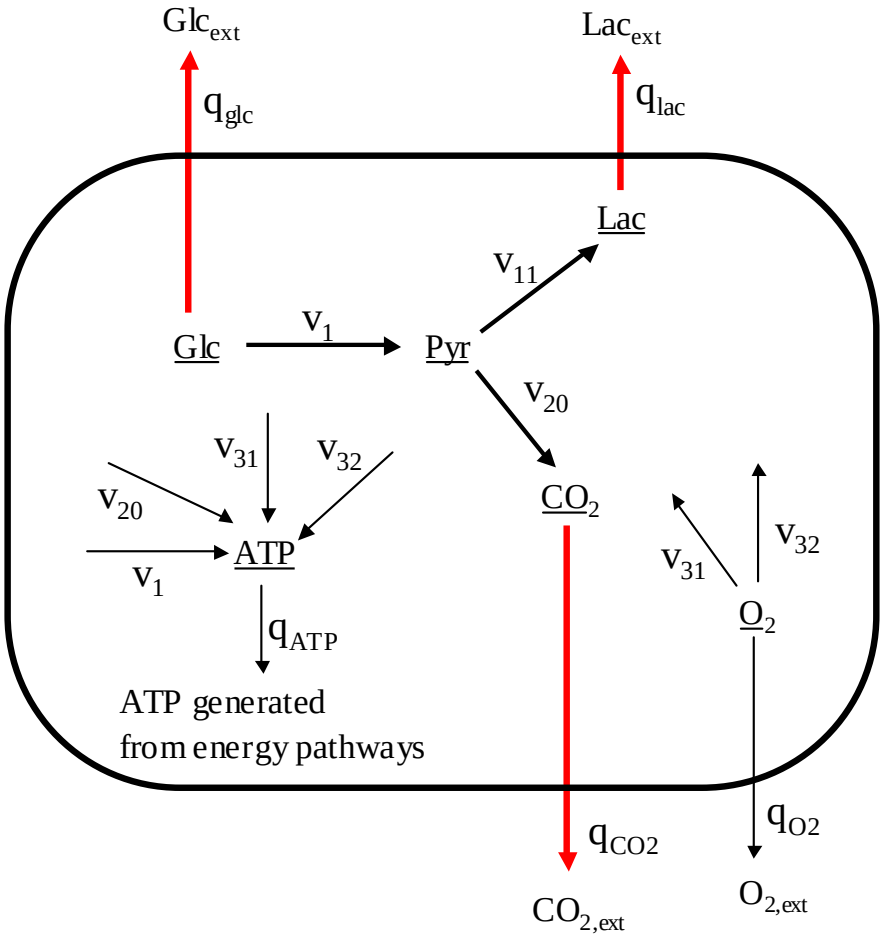
The Glc/Lac/CO_2 well-plate protocol



Changes in concentrations of glucose, lactate, and carbon dioxide (CO_2 plus bicarbonate) are used to calculate the respective metabolic rates

Metabolic flux analysis



Balances for NADH and $FADH_2$ not shown

Given measured rates, we calculate the least-square values for the unknown fluxes.

v1 ~ glycolysis
v20 ~ TCA cycle
v31 and v32 ~ oxidative phosphorylation
qATP ~ energy production

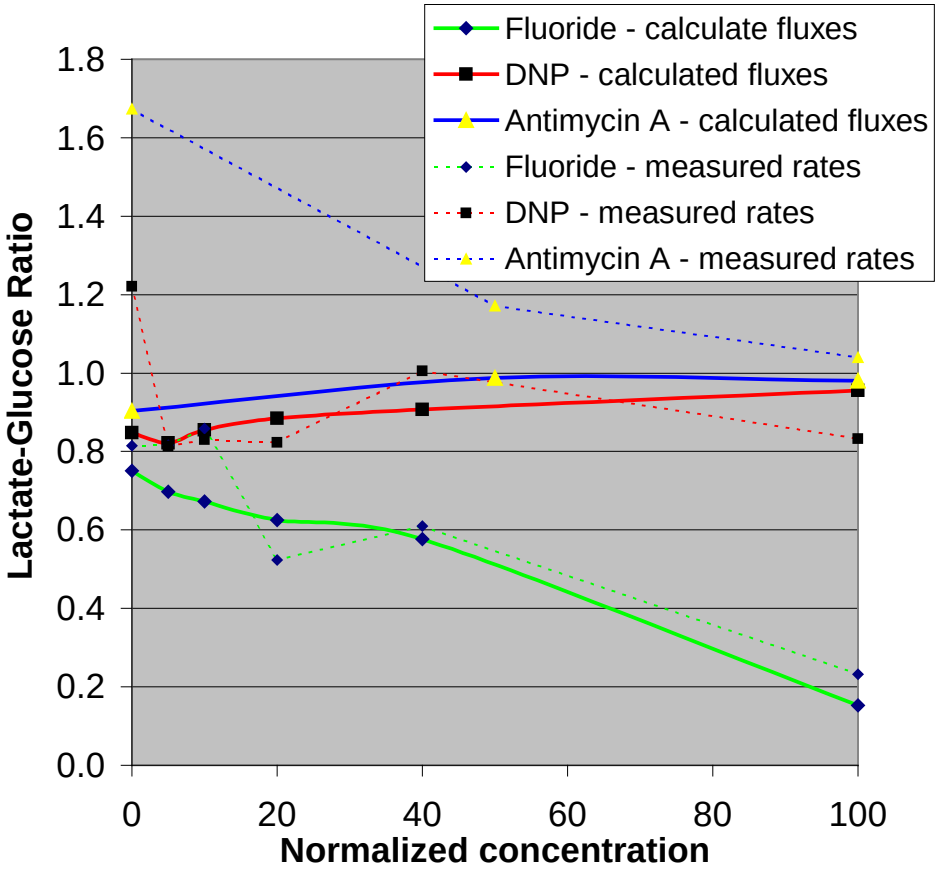
Balances on pathway intermediates:

Glc	**-1**	**0**	**0**	**0**	**-1**	**0**	**0**	**0**	**0**	**0**
Lac	**0**	**-1**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**
CO2	**0**	**0**	**-1**	**0**	**0**	**0**	**3**	**0**	**0**	**0**
O2	**0**	**0**	**0**	**-1**	**0**	**0**	**0**	**-0.5**	**-0.5**	**0**
Pyr	**0**	**0**	**0**	**0**	**2**	**-1**	**-1**	**0**	**0**	**0**
NADH	**0**	**0**	**0**	**0**	**2**	**-1**	**4**	**-1**	**0**	**0**
FADH2	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**-1**	**0**
ATP	**0**	**0**	**0**	**0**	**2**	**0**	**1**	**2.5**	**1.5**	**-1**

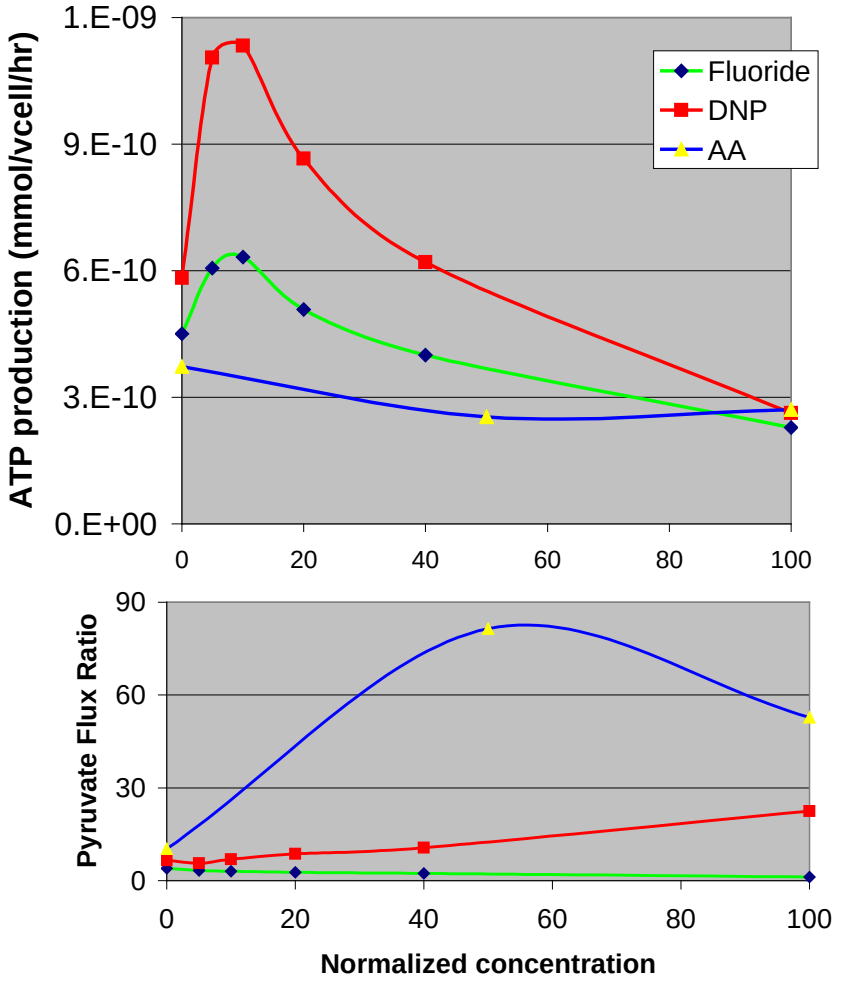
$* \begin{pmatrix} \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \end{pmatrix} = \underline{0}$

Sample results from metabolic flux analysis

Fluxes have reduced noise



ATP estimates and Pyruvate Node provide clear biosignatures



Metabolic Screening of Cell Lines using Well-Plates

- **Preliminary screening can be accomplished using the 90-minute acidification well-plate assay**:
  - Establish working ranges for toxins on cell lines
  - Assess cytotoxicity upon 90-minute exposures
- **Metabolic changes can be quantified using a composite protocol where each of 400-ul mammalian cell cultures on a 24-well plate provides glucose, lactate, and CO2 metabolic rates:**
  - Plug design allows for monitoring of net CO2 produced
  - Specifying 3 metabolic rates provides an overdetermined system of linear equations for the metabolic network
    - Each well can be tested for consistency of measurements with the model (data error exclusion)
    - MFA calculations results in least-square estimates for 10 fluxes (noise reduction)
- **Future development of oxygen well-plate assay for oxygen metabolic rates:**
  - Provide alternate fast (90-minute) screen for toxin concentration range
  - Direct assessment of toxin impact on oxidative phosphorylation

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  Modified Cytosensor MicroPhysiometer

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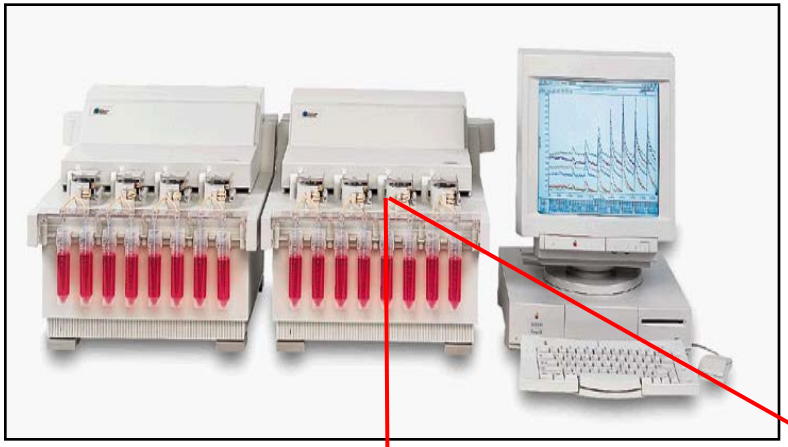
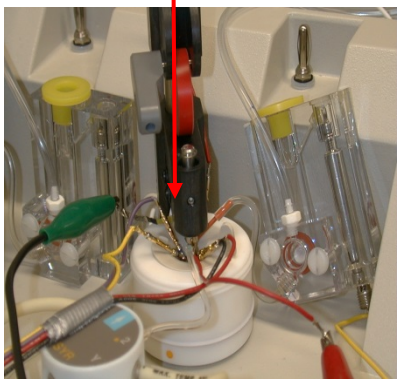
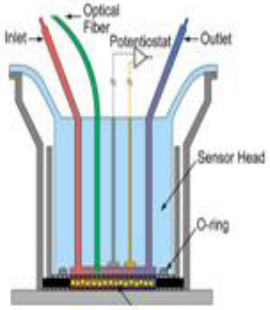
BioSignature Generation

Cell Line Selection, Cell Conditioning, Sensor Array Optimization

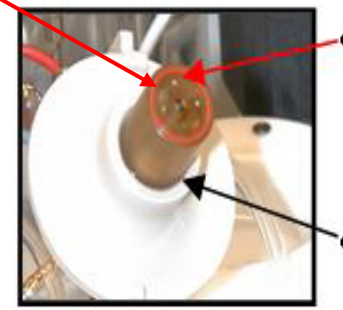




MicroPhysiometer: Modified sensor head



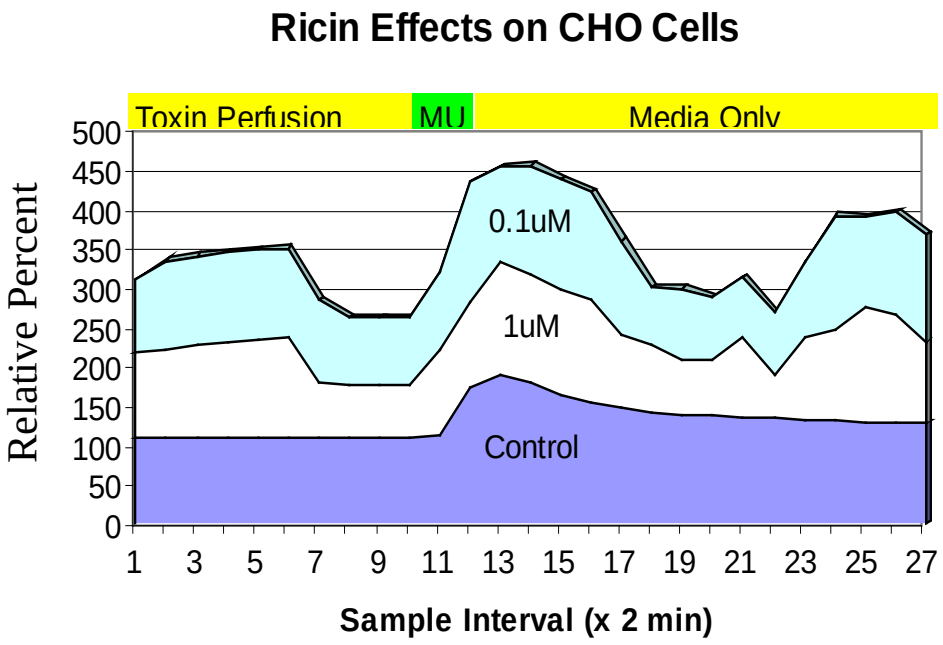
Schematic drawing of modified sensor head for the microliter Molecular Devices Cytosensor microphysiometer

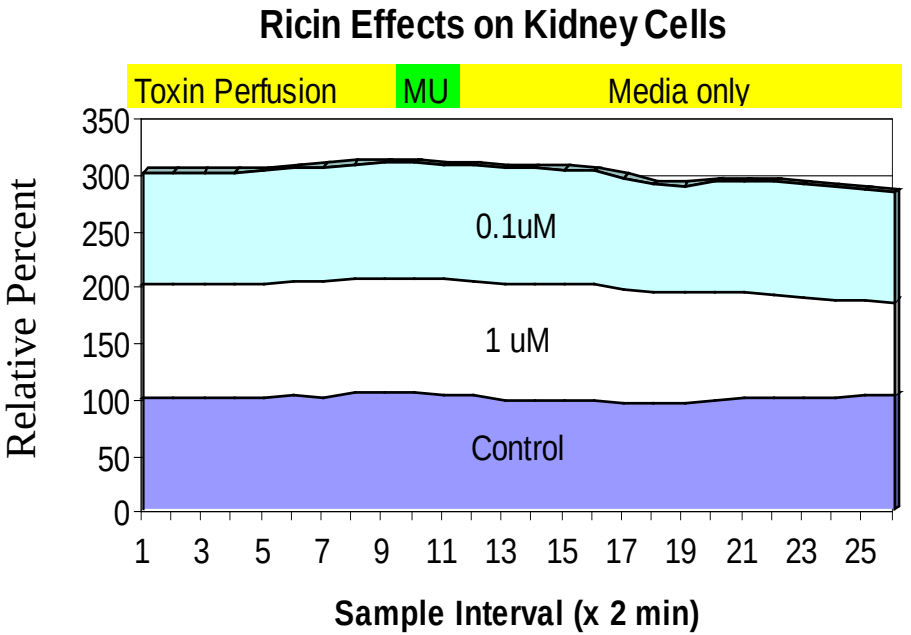


- Four new platinum electrodes for oxygen, glucose, lactate, ORP measurements
- Existing Cytosensor head

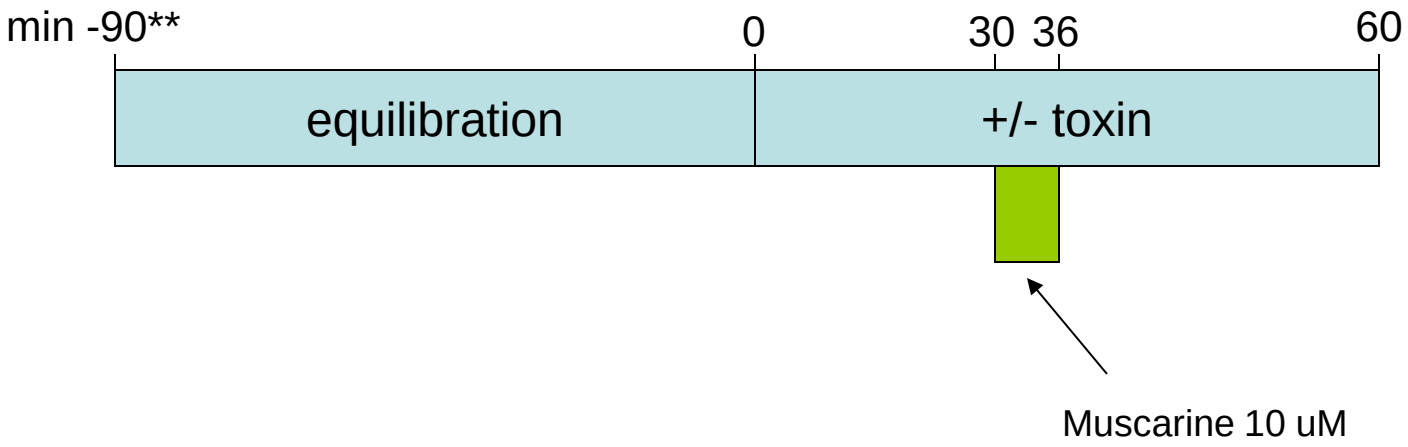
Acidification Effects of Ricin

- ECBC/SCMCOM
- Toxin perfusion (20 minutes)
  - 0.1 uM
  - 1 uM
  - Control
- Muscarine stimulation
- Washout
- CHO and kidney cells,





General CytoSensor MicroPhysiometer Protocol

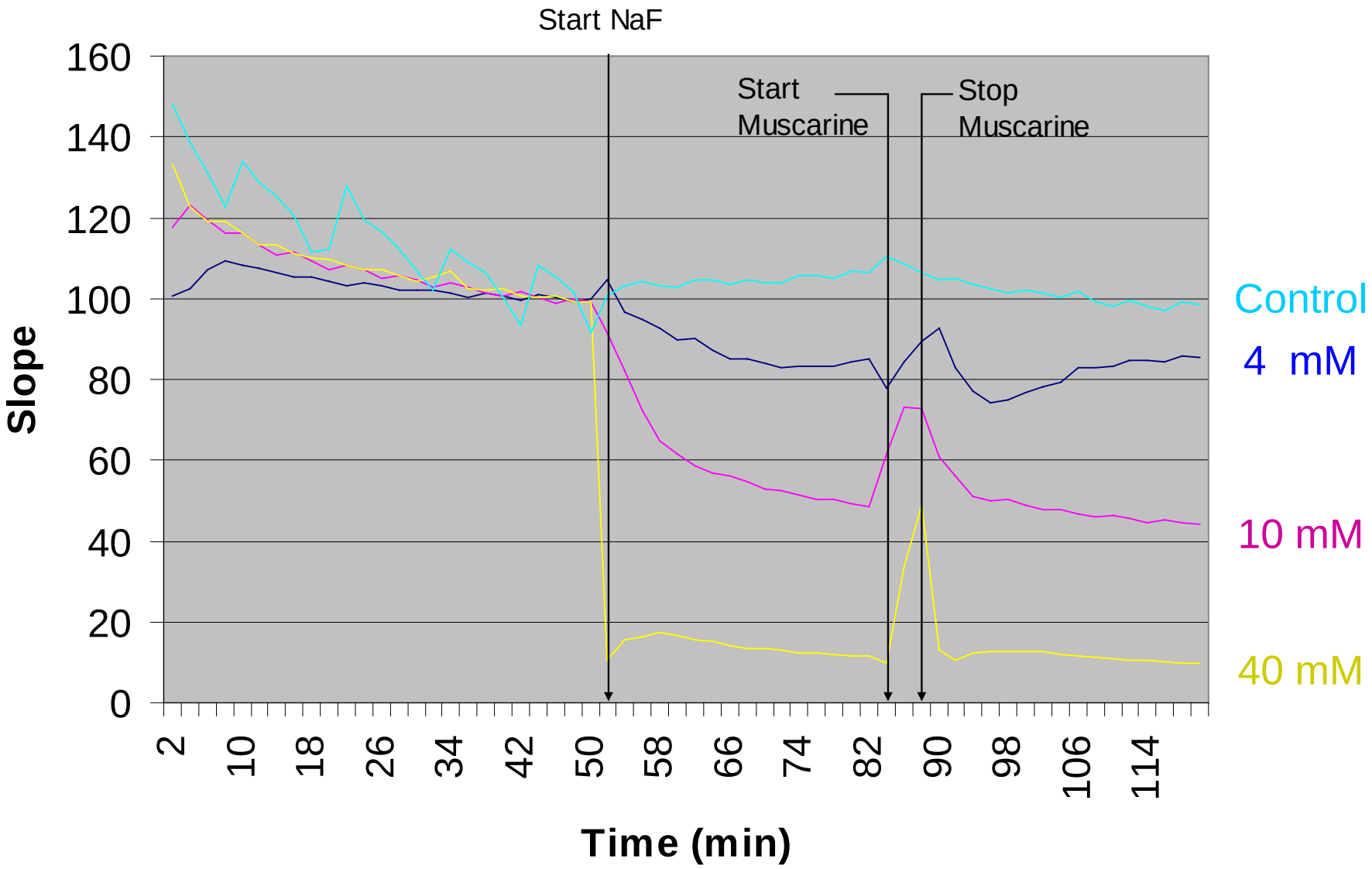


Pump measurement settings on a 2 minute cycling time 2 sec delay 30 sec measurement

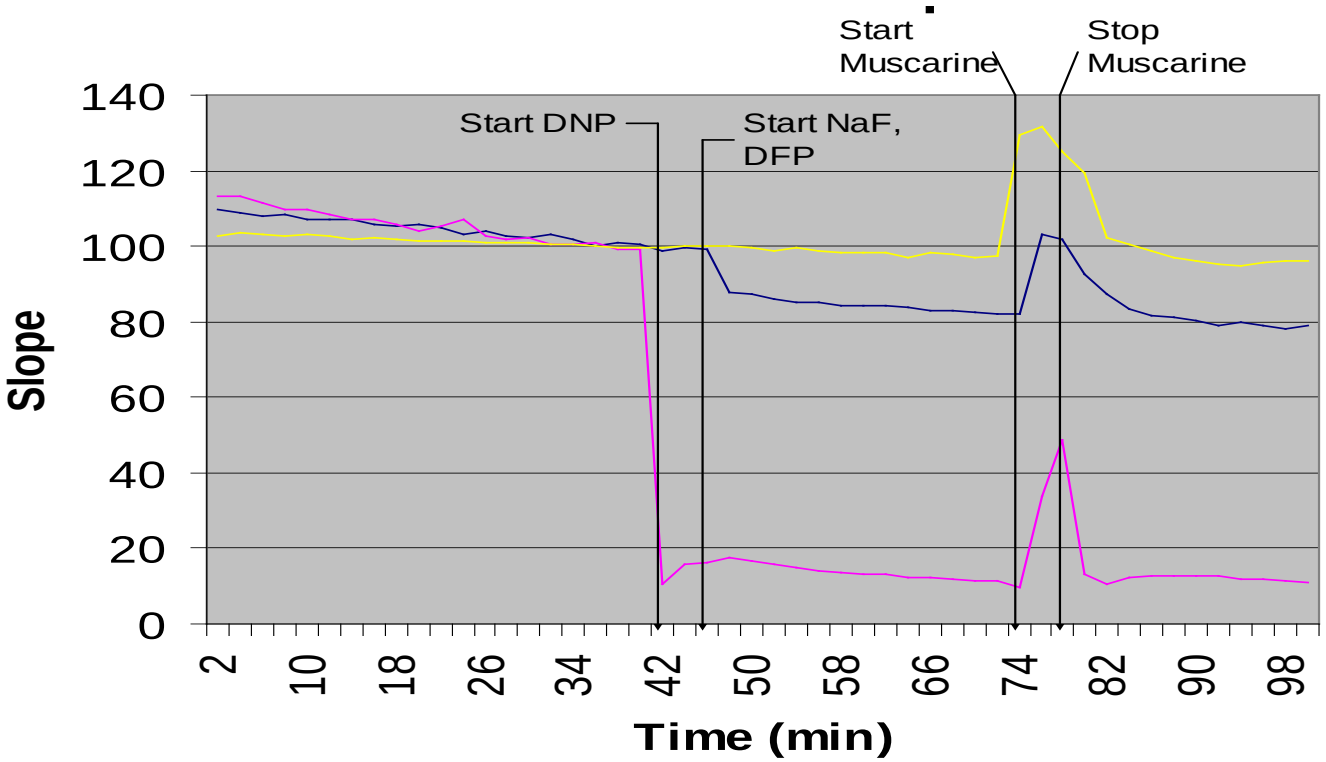
Cell Lines:
CRL 2254 (AML12) Mouse liver cell
CRL-1981 M3WT4 Chinese Hamster Ovary
CRL-10225 Fibroblast

Bio-Signature is Dose Dependent

Fibroblasts in NaF



A Family of Bio-Signatures

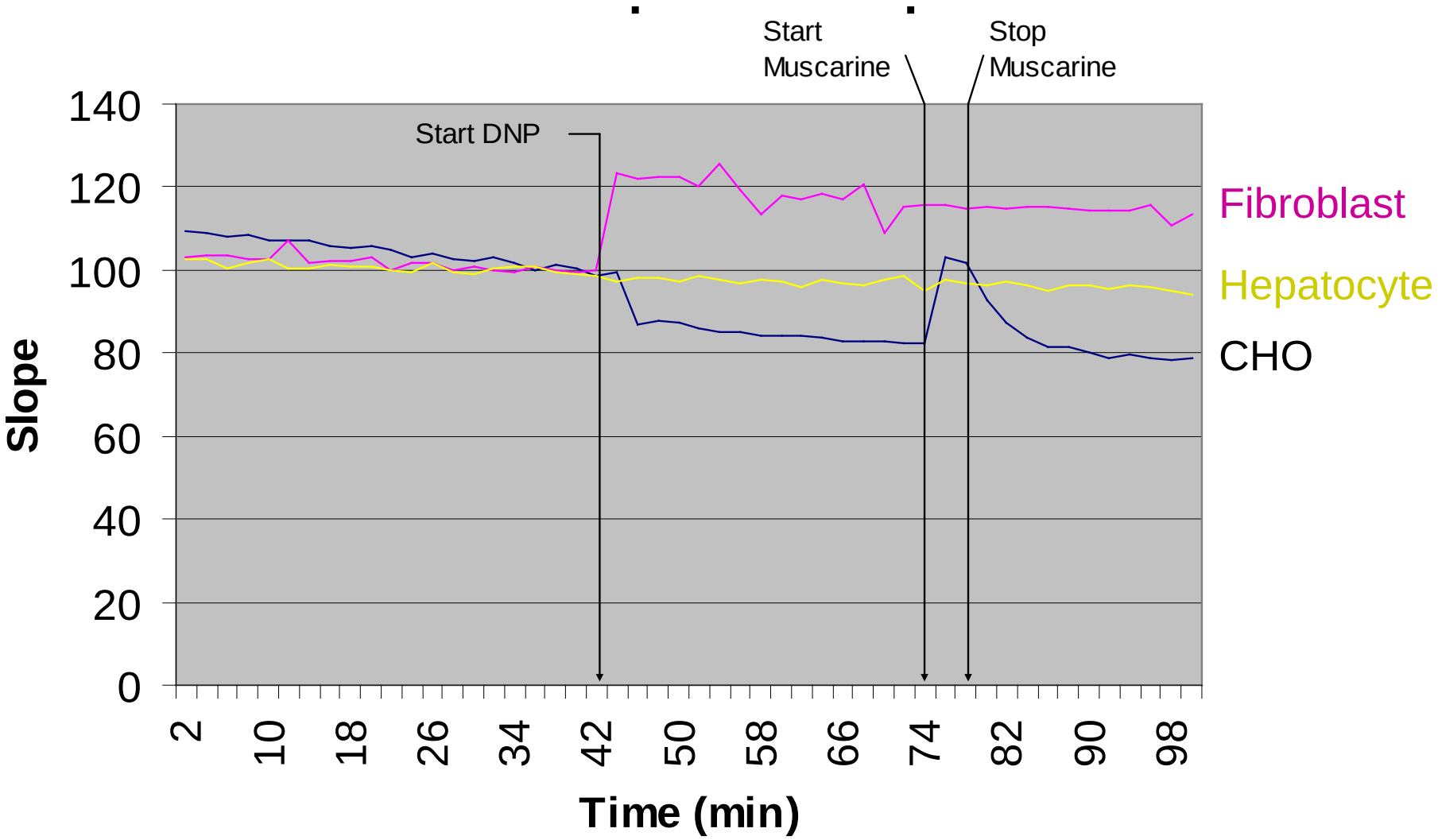


CHO 1.0 uM DFP

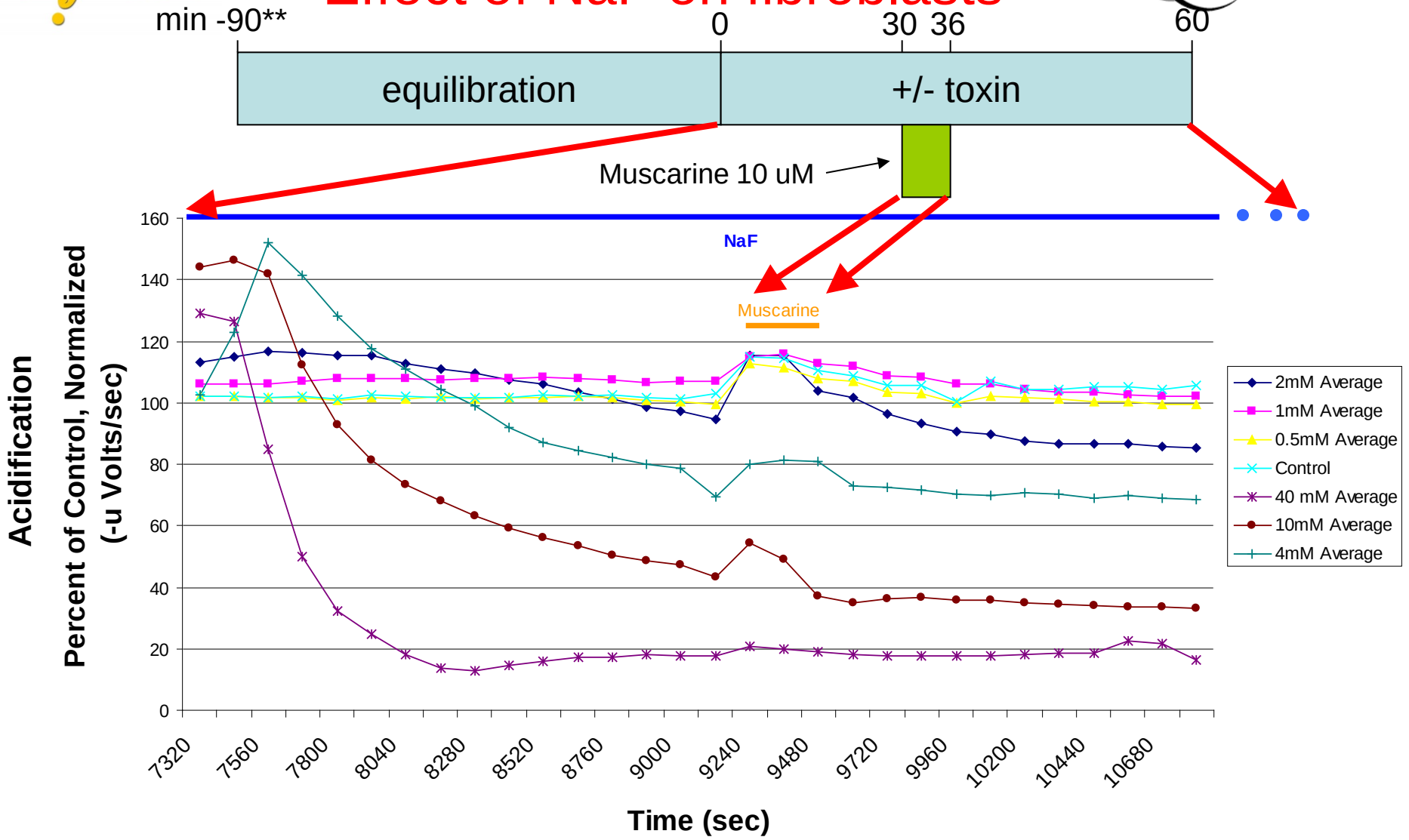
CHO 0.1 uM DFP

FIB 40 mM NaF

Bio-Signature is Cell Specific



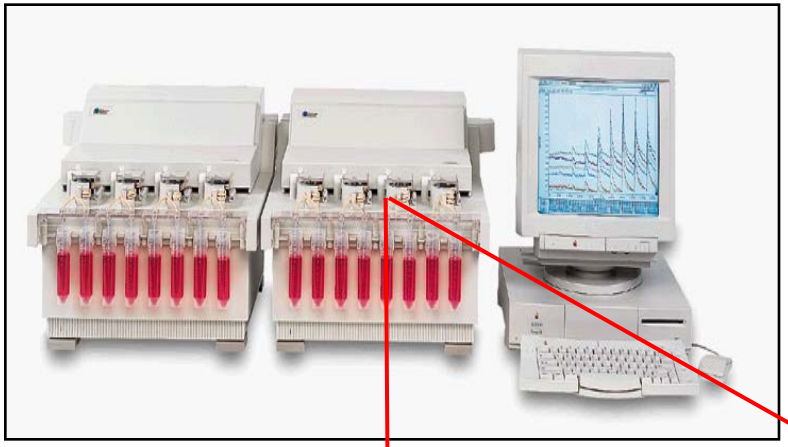
Effect of NaF on fibroblasts

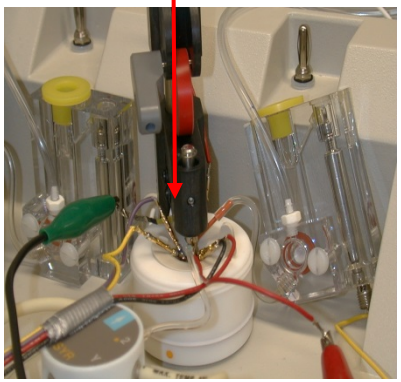


First data point is the first data measurement taken after beginning infusion of drug. All data expressed relative to normalized segment of data over the 10 minutes immediately prior to drug infusion. Muscarine injected at 10uM.

BioSignature Generation:

Cell Line Selection, Cell Conditioning, Sensor Array Optimization





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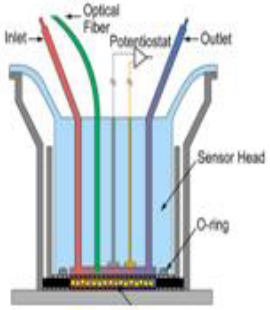

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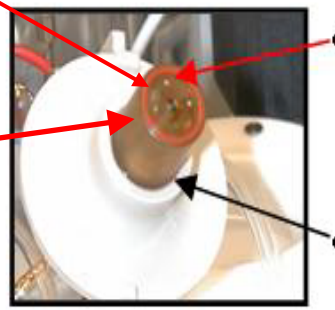


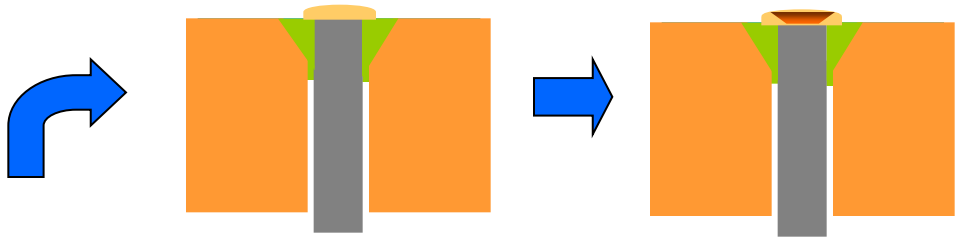
- Four new platinum electrodes for oxygen, glucose, lactate, ORP measurements
- Existing Cytosensor head

Fabrication of LOx and GOx Sensor Head Electrodes

LOx electrode

Deposit LOx/Glutaraldehyde/BSA film

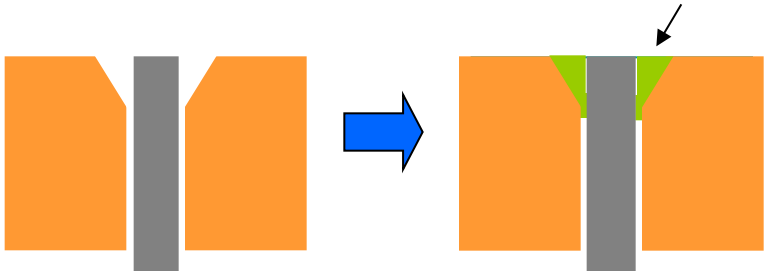
Deposit 0.5% Nafion



Sensor Head Electrode

Insert Pt electrode into sensor head

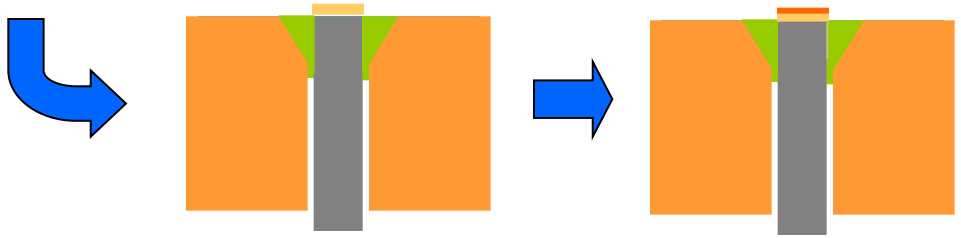
Seal with epoxy/polish



GOx electrode

Deposit GOx/Glutaraldehyde/BSA film

Deposit 5% Nafion



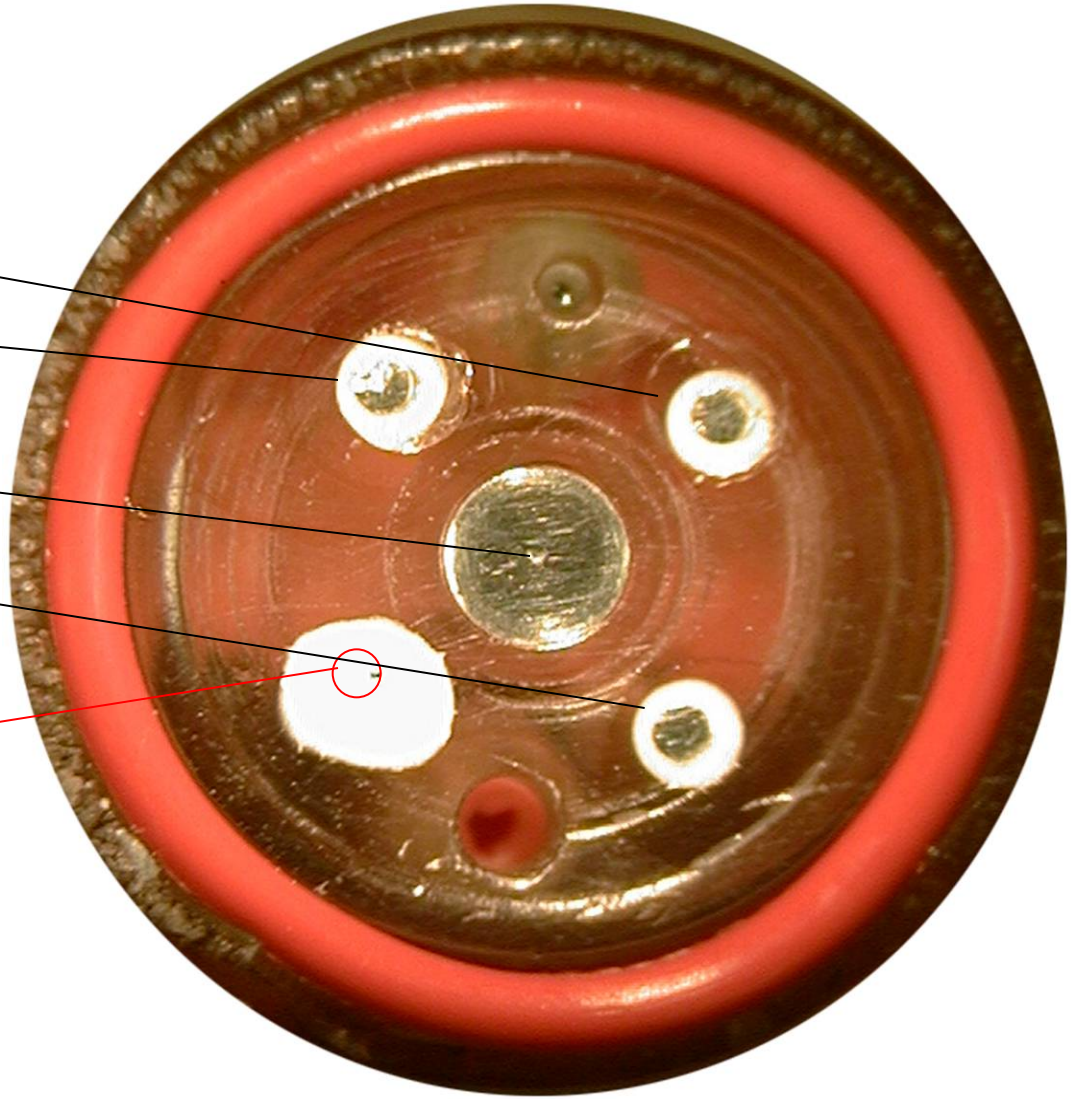
Platinum Electrodes in Modified CytoSensor Head

510 um diameter

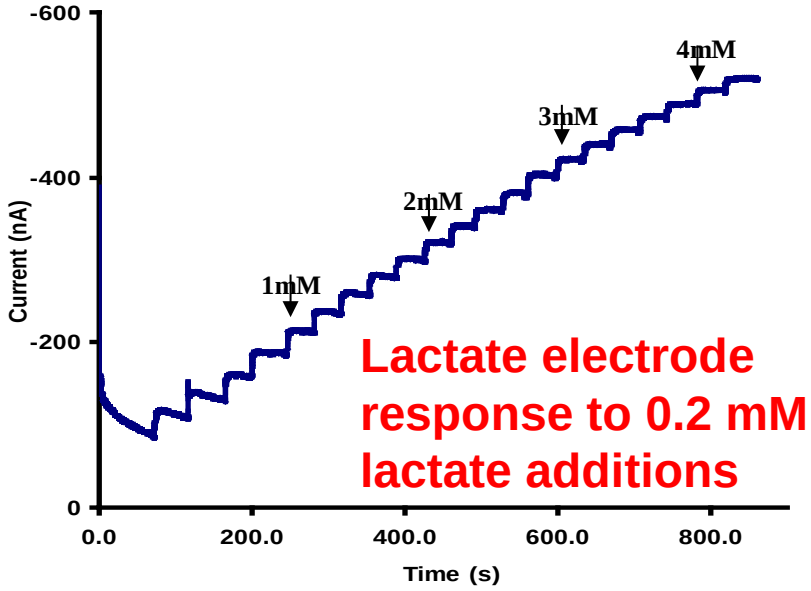
- Glucose
- Lactate
- pH
- Reference

127 um diameter

- Oxygen

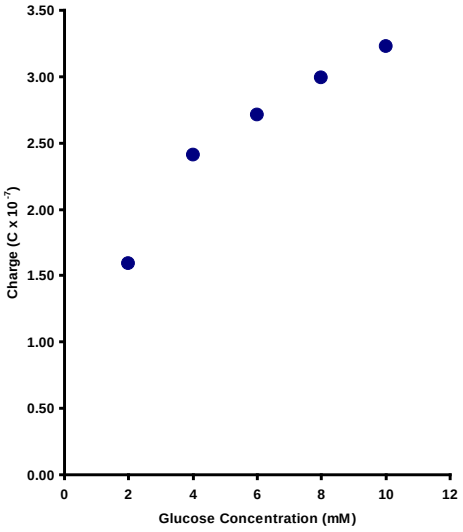


Glucose and Lactate Sensor Calibration in the Microphysiometer (No Cells)

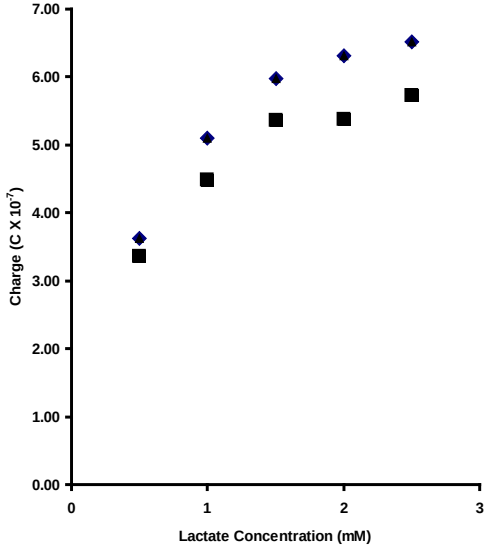


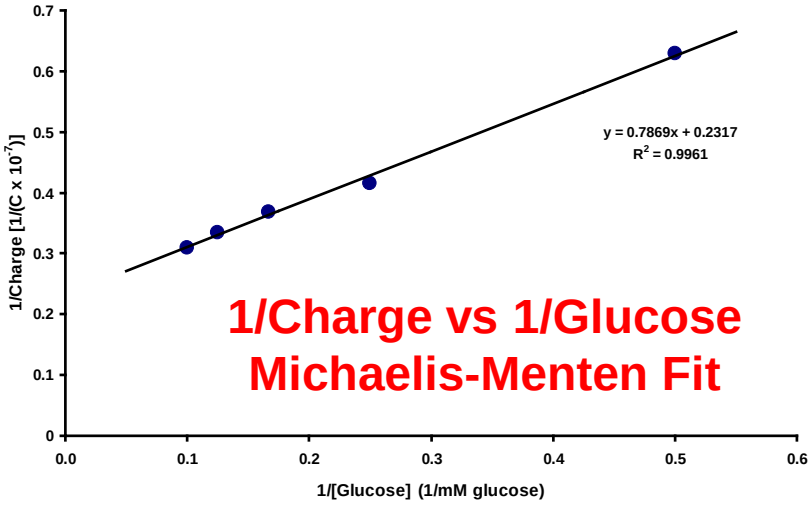
Lactate electrode response to 0.2 mM lactate additions

Charge vs Glucose

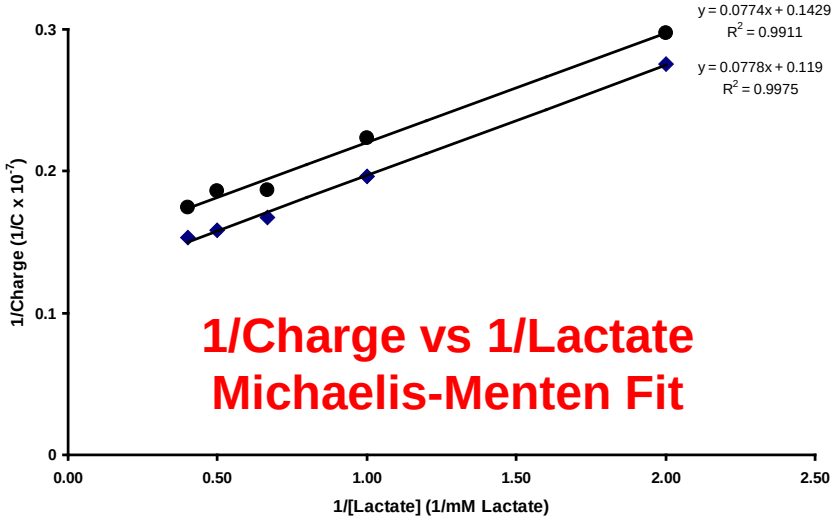


Charge vs Lactate



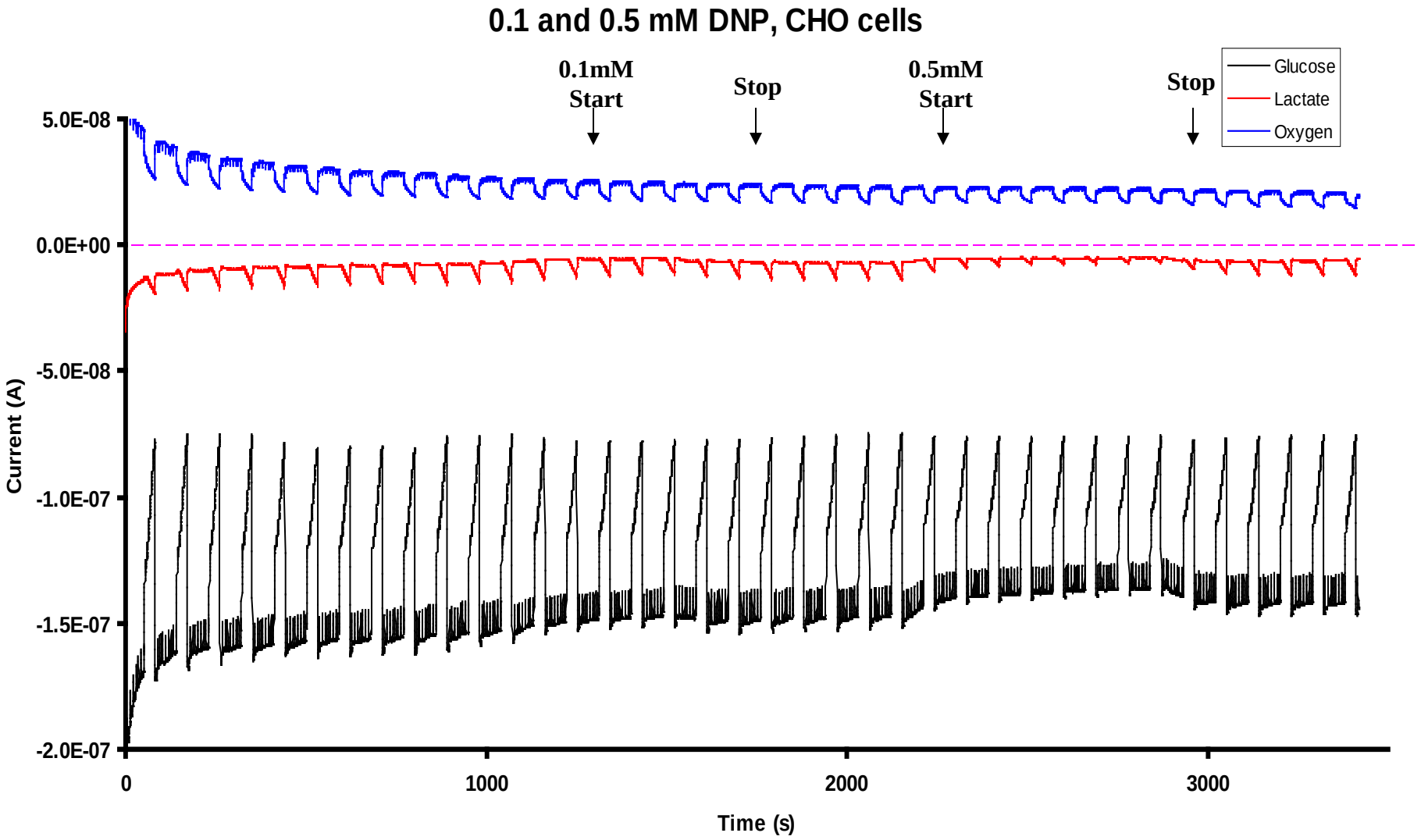


1/Charge vs 1/Glucose Michaelis-Menten Fit



1/Charge vs 1/Lactate Michaelis-Menten Fit

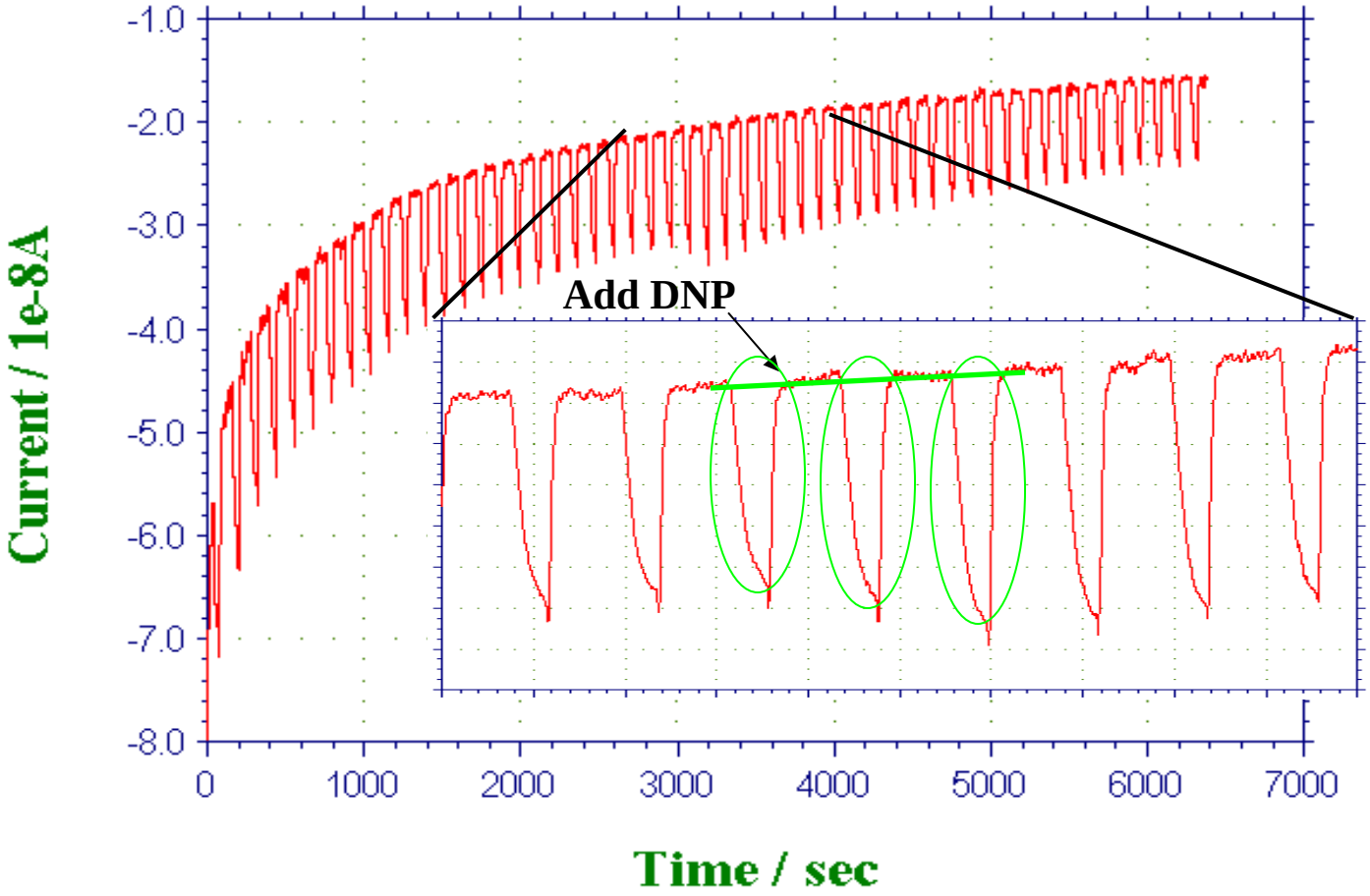
Raw data for 0.1 and 0.5 mM DNP, CHO cells



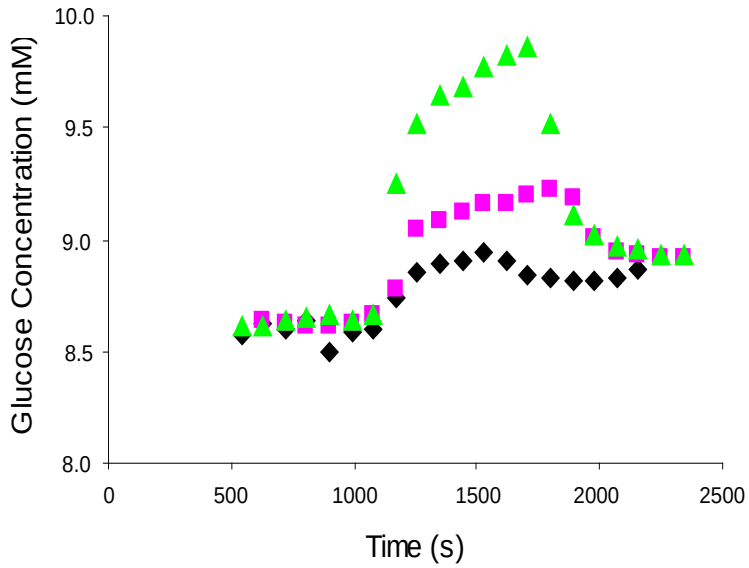
Data Analysis: Integration below baseline

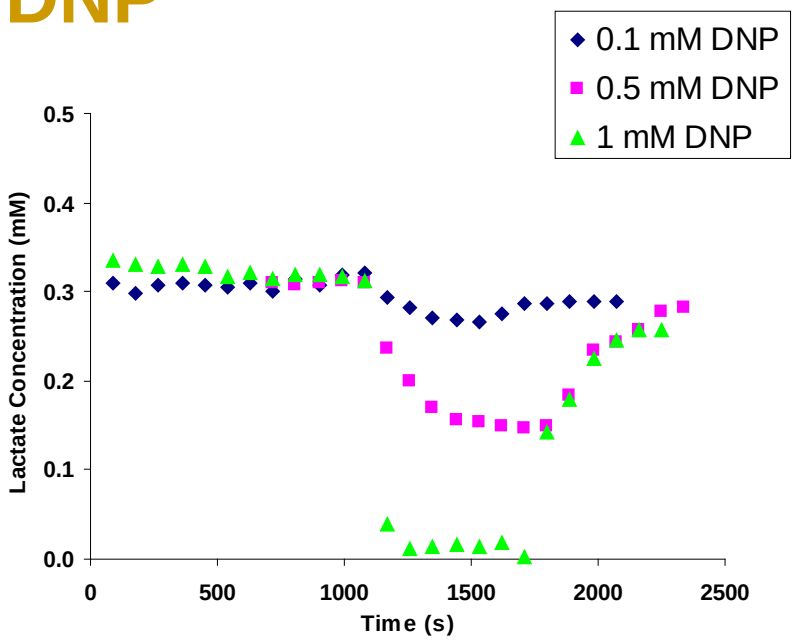
Cellular Lactate Response to 20μM DNP in RPMI

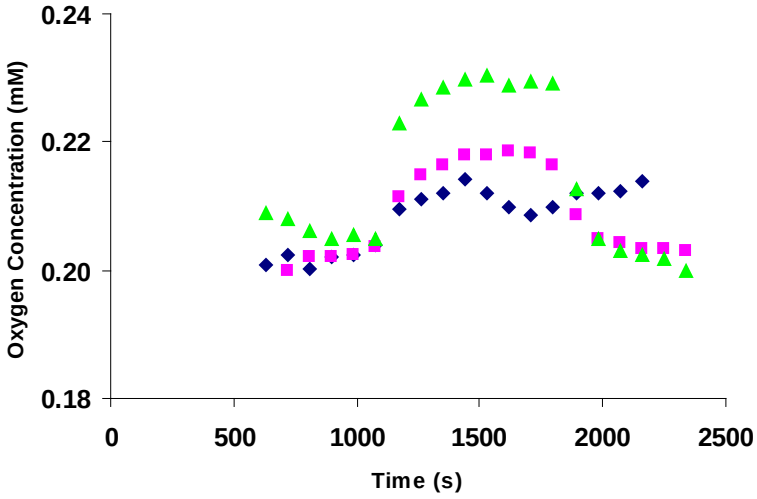
LOx/BSA/TritonX electrode (Raw data)

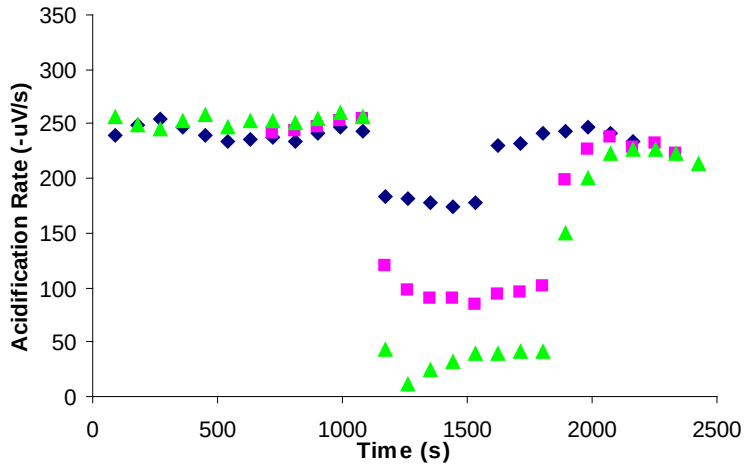


Dose response with 0.1, 0.5, and 1 mM DNP

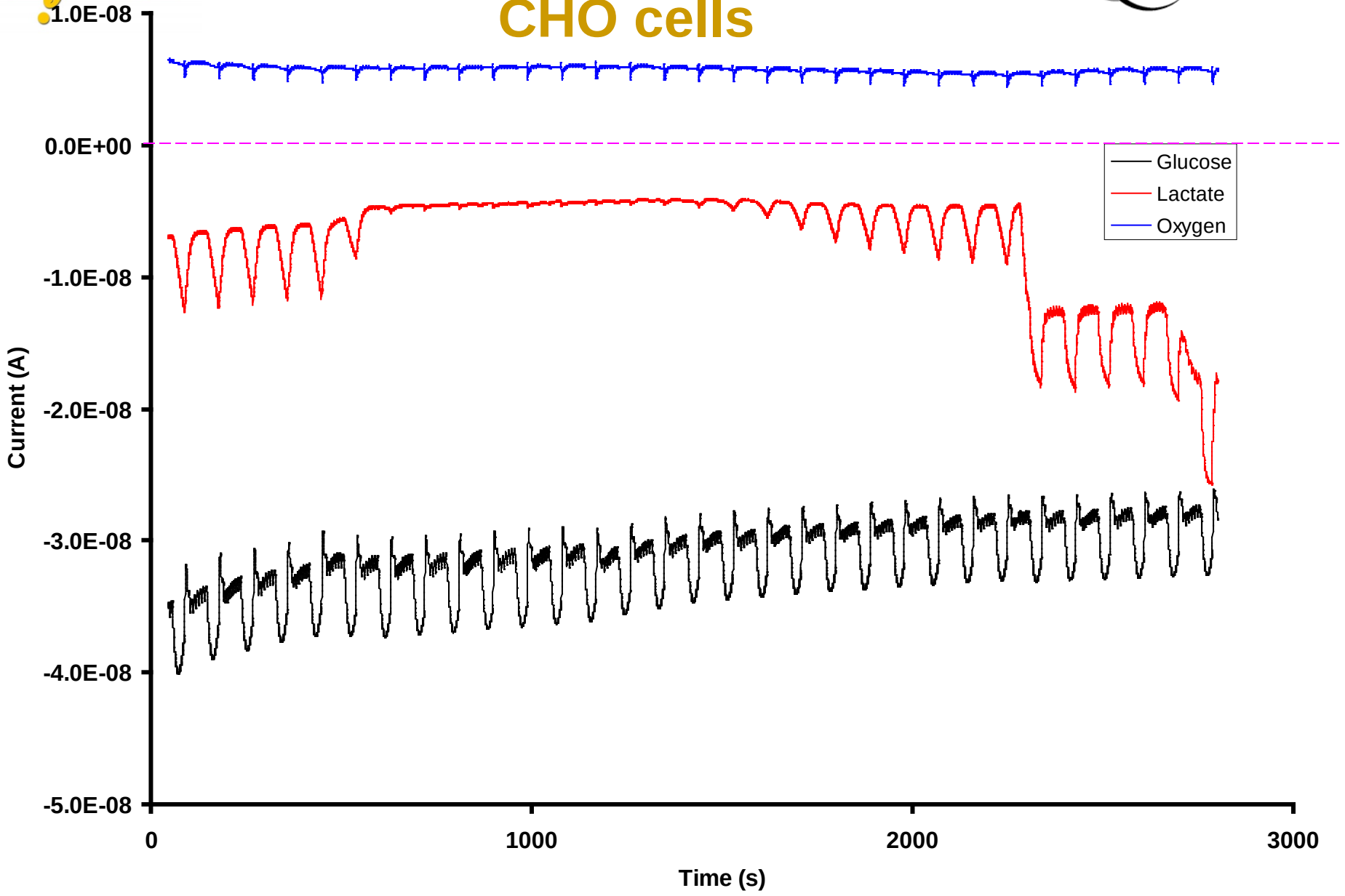




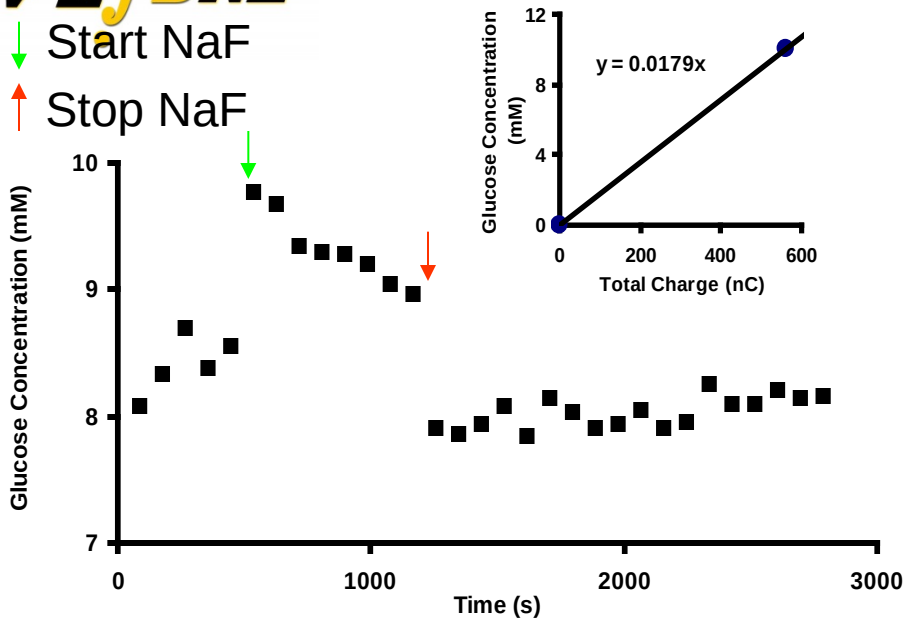


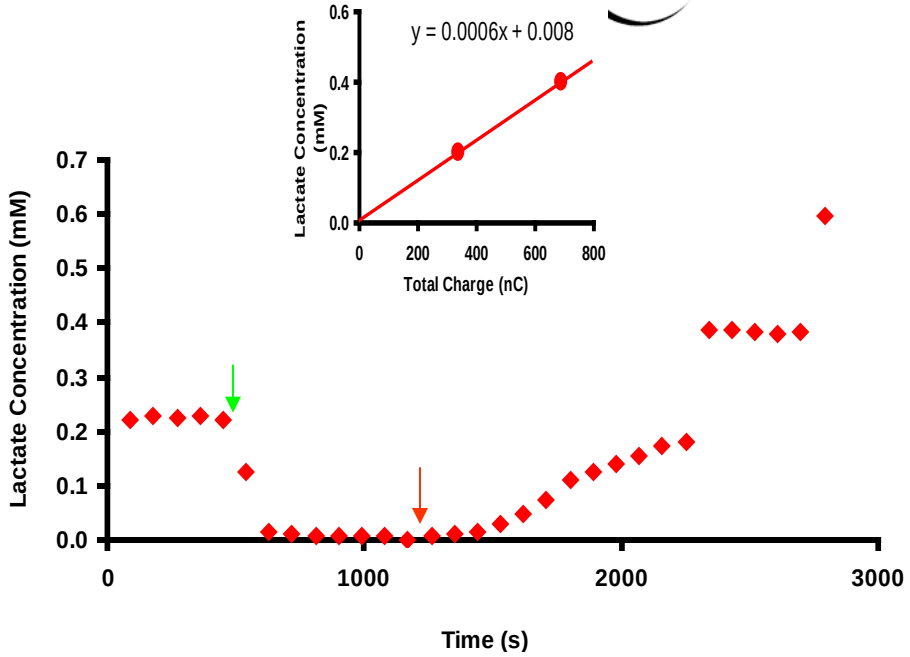


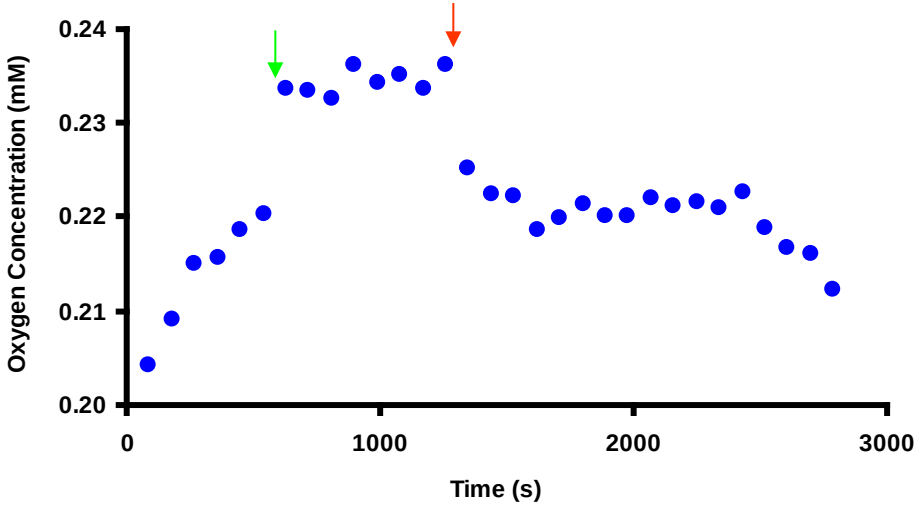
Raw data for 20 mM NaF on CHO cells

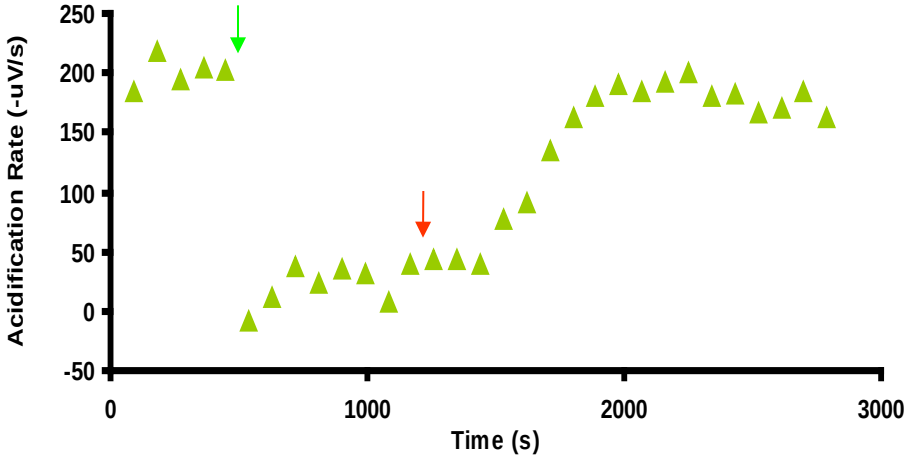


Effect of 20 mM NaF on CHO cells



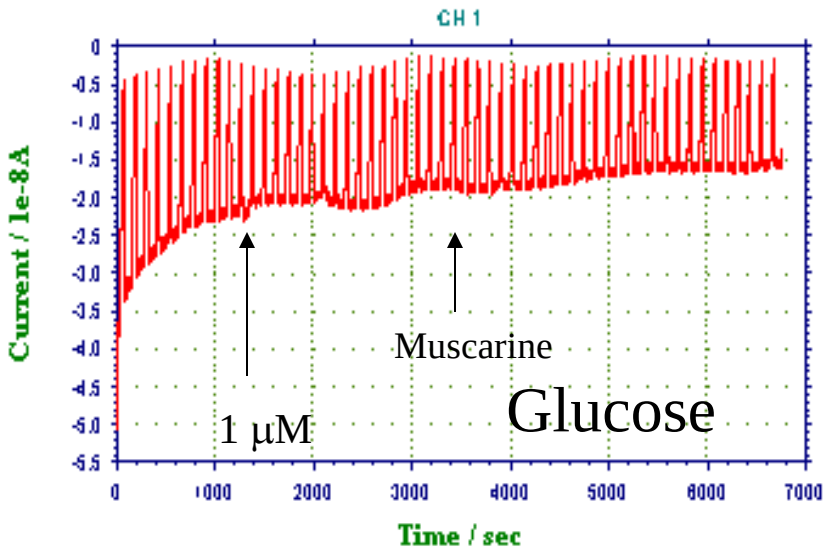


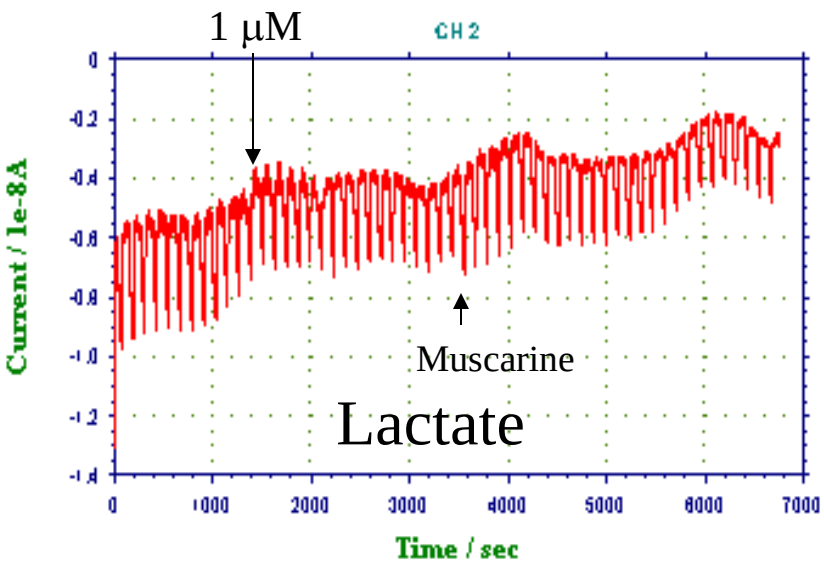


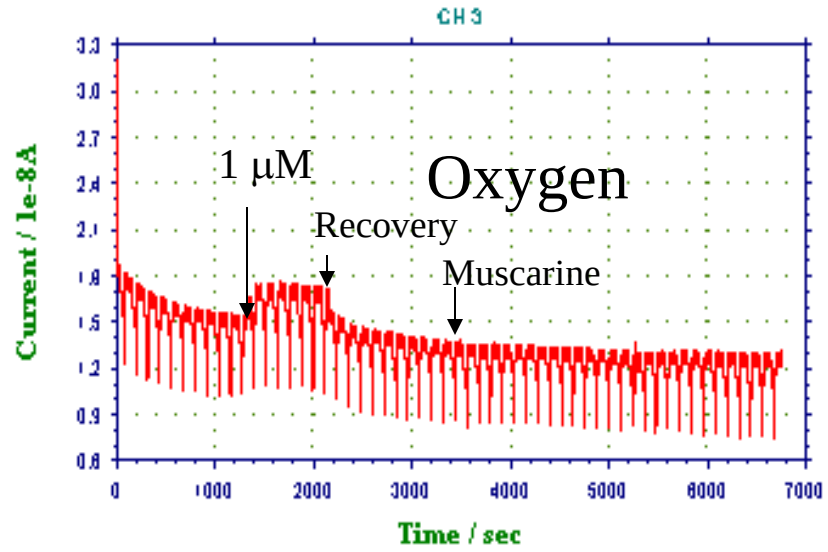


- **Includes 2 point t calibration for Glucose, Lactate**
- **Oxygen concentration estimated at 10% depletion with “normal” cells (before toxin addition)**

Modified CytoSensor Microphysiometer Head at ECBC







CHO Cells response to 1 uM Cholera Toxin, followed by Muscarine Stimulation

Three Spatial/Temporal Scales for Cell-Line Screening and BioSignature Generation

- Milliliter – minutes to an hour

  96 and 24-well-plate cell culture

- Microliter – 10-100 seconds

  Modified Cytosensor MicroPhysiometer

- SubNanoliter – 10-100 milliseconds

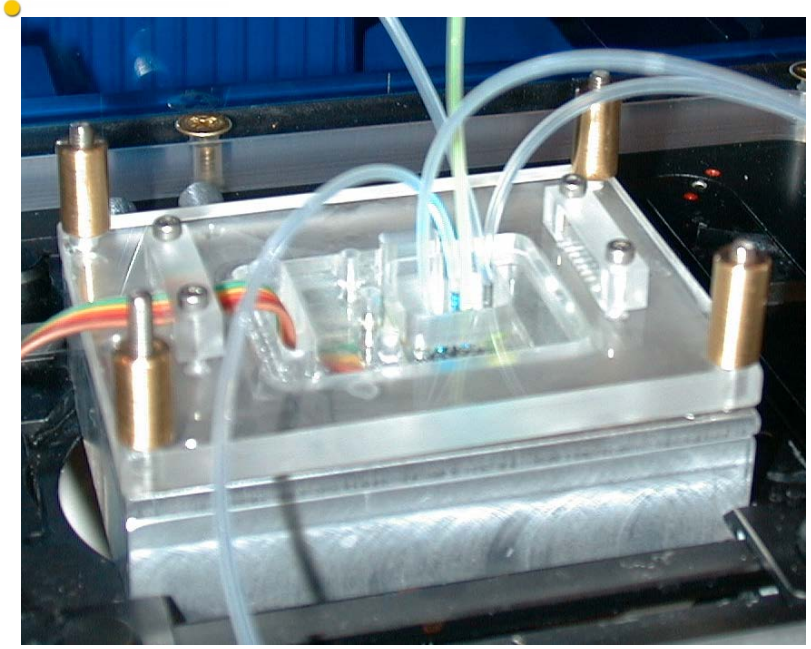
  Vanderbilt NanoPhysiometer

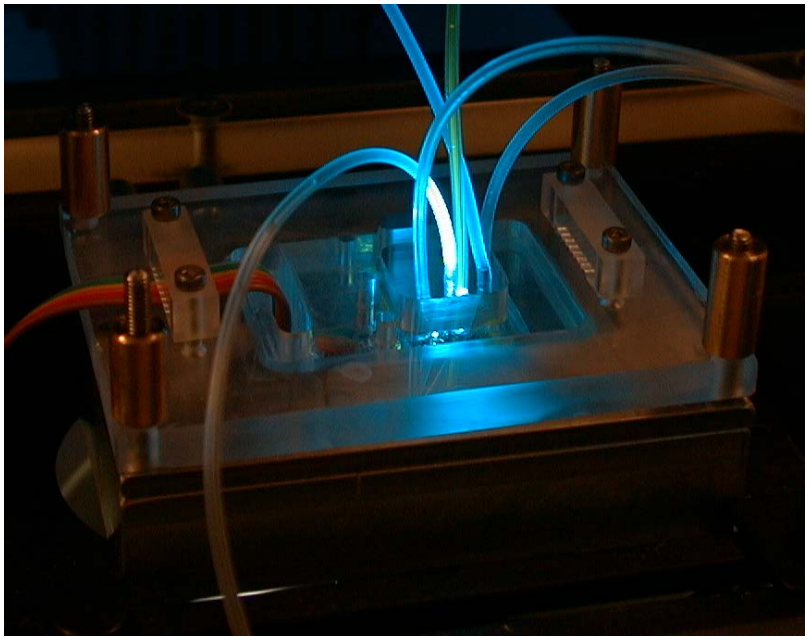
NanoPhysiometer

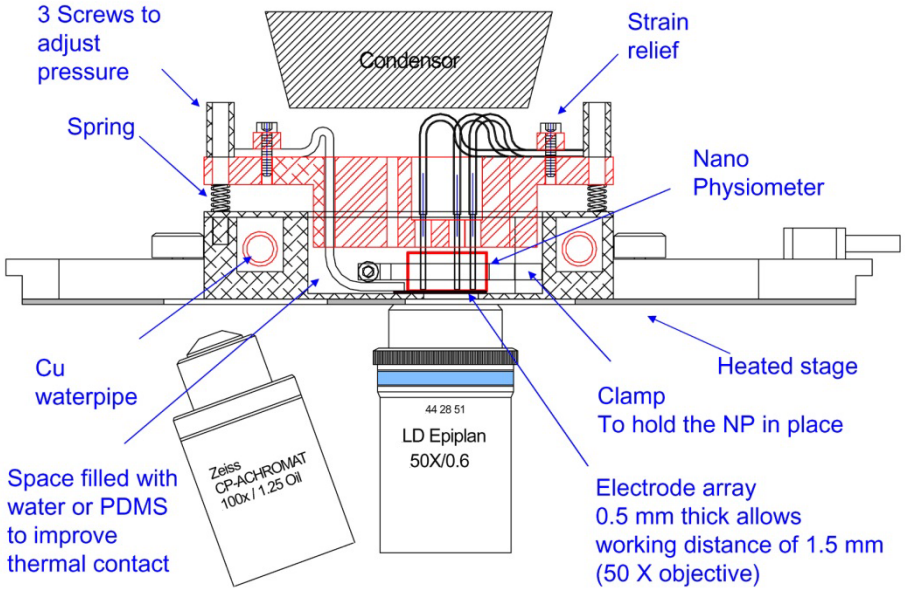
- Both gain and bandwidth contribute to clearer biosignatures and hence improved discrimination
  - The physiological response provides the gain
  - A single cell in a small volume provides the bandwidth!

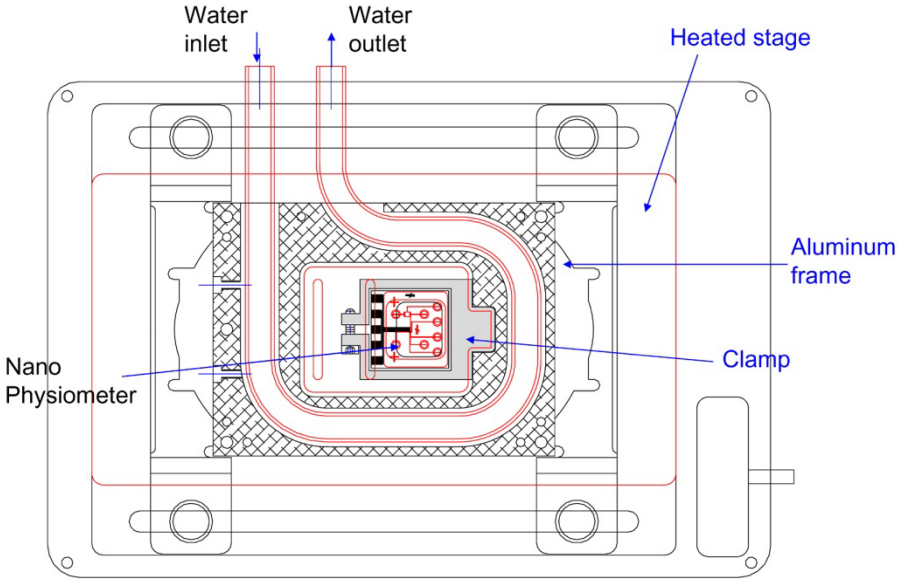
➡ Monitor single-cell metabolic physiology in real time in sub-nanoliter volumes

Nanophysiometer III: MicroIncubator



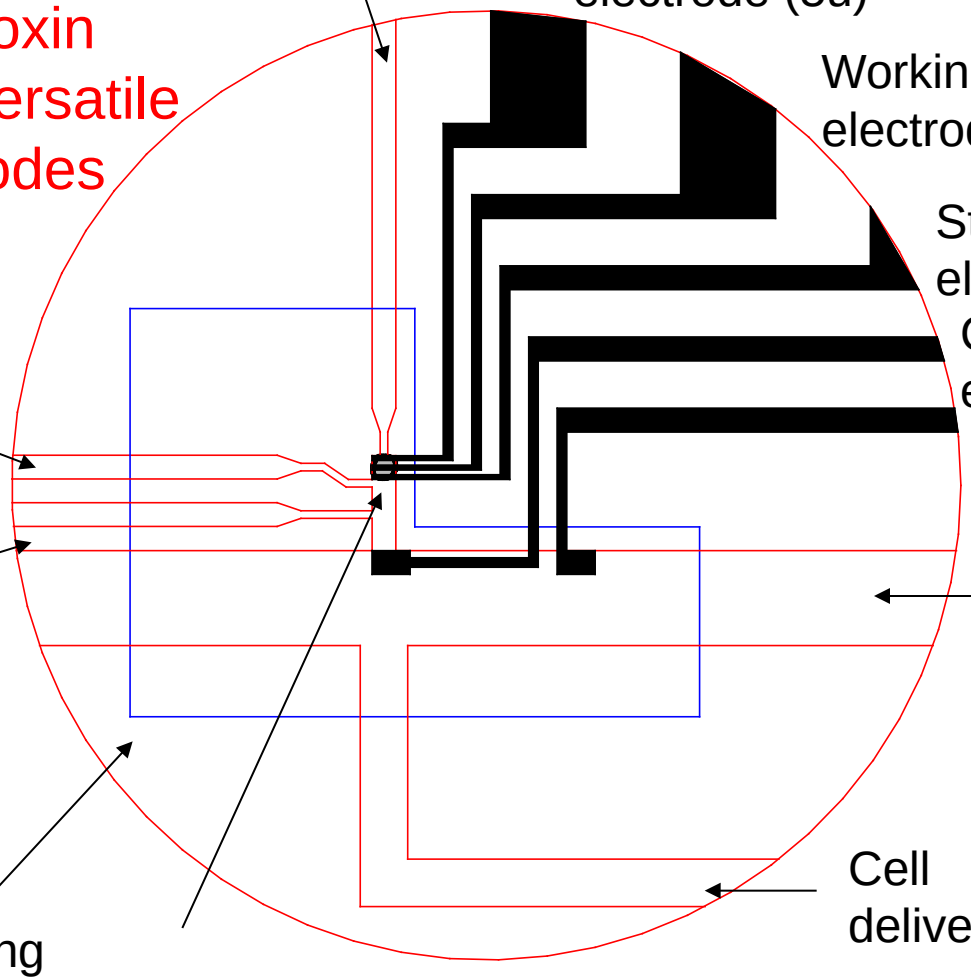




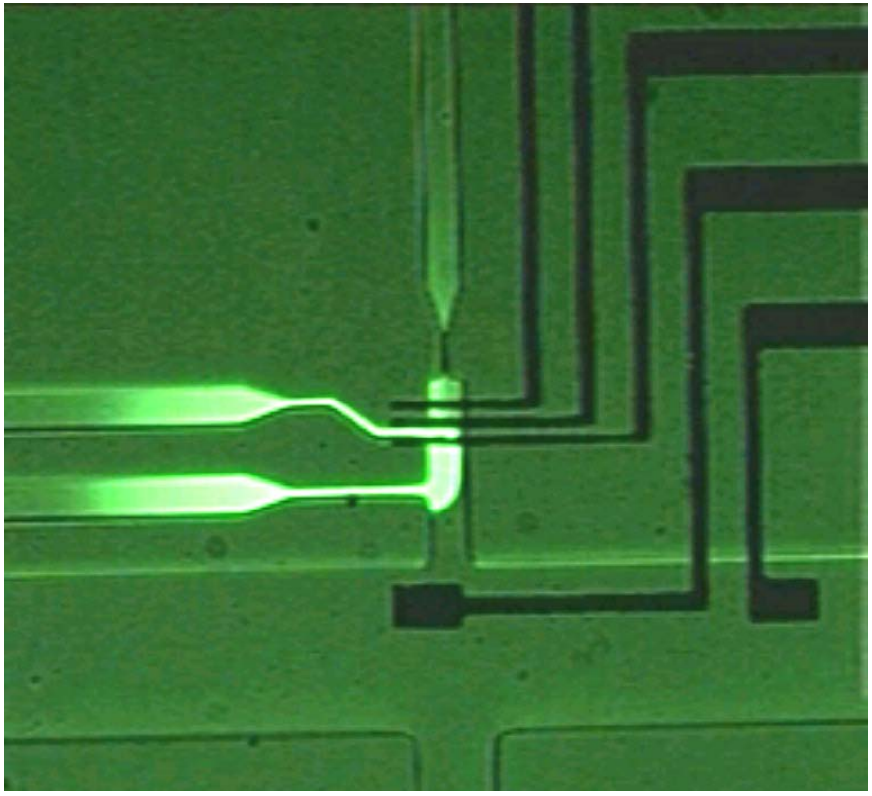


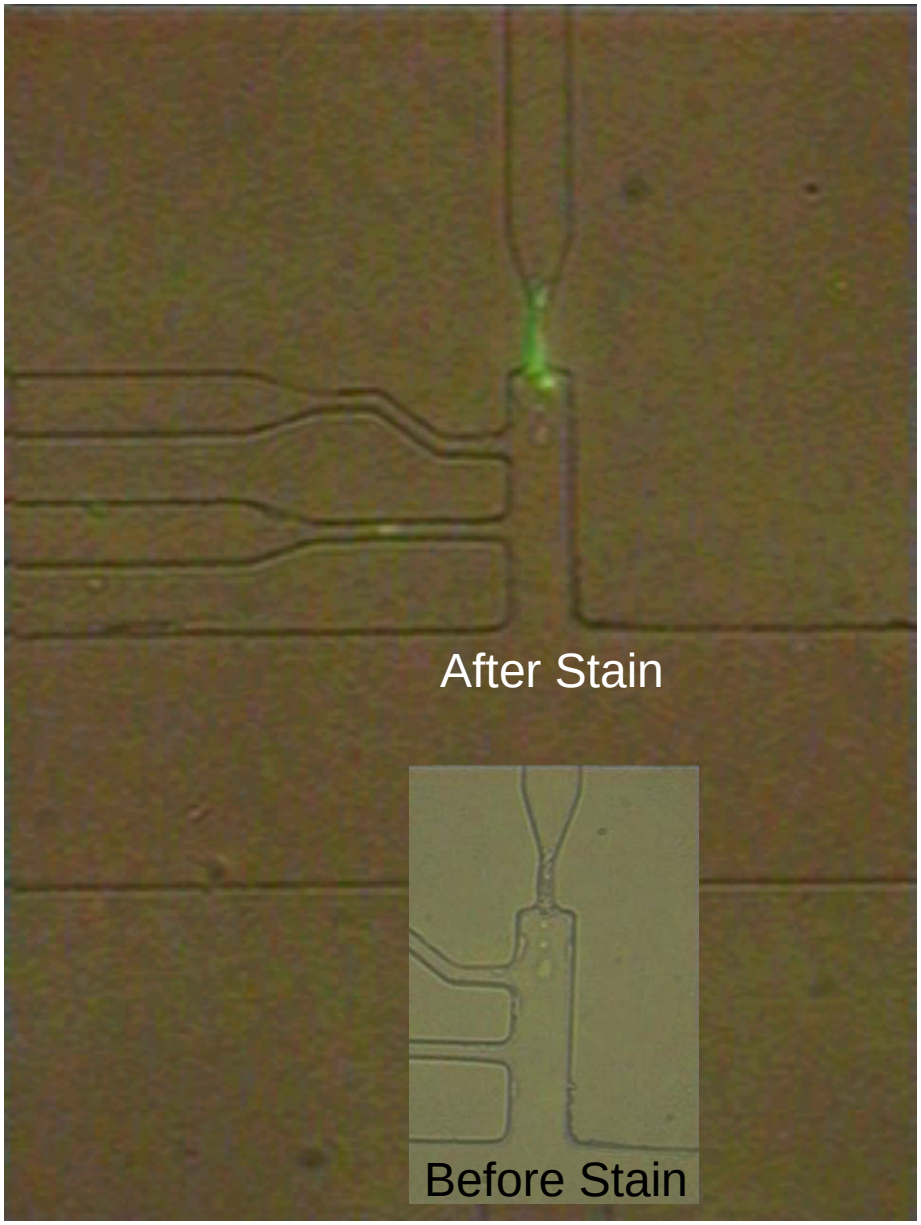
Nanophysiometer III

Expanded view of a cell trap with toxin delivery and versatile custom electrodes

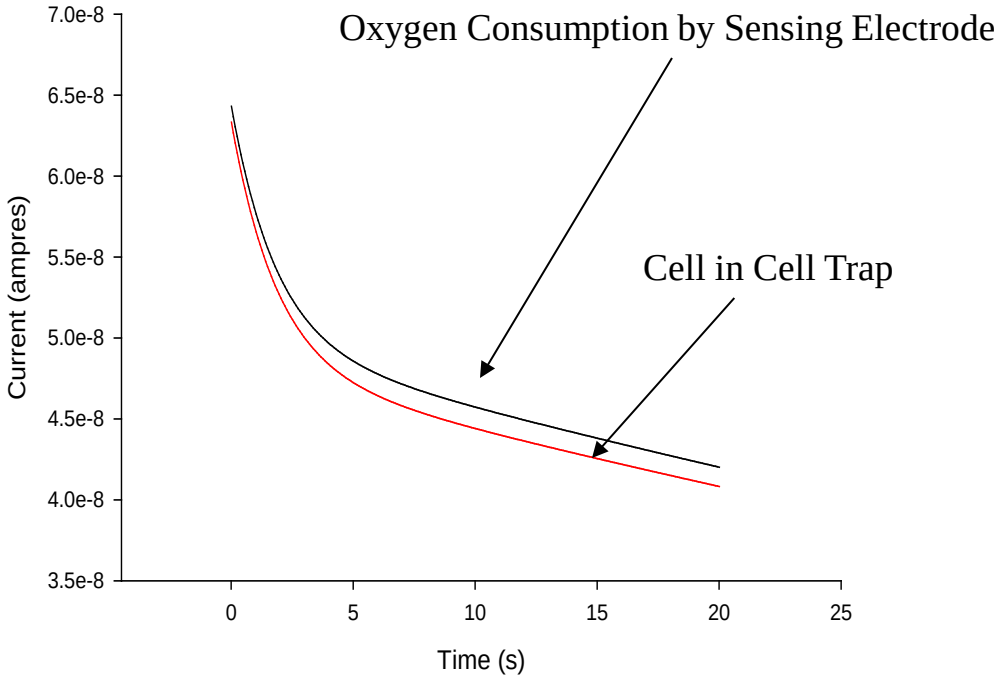


NanoPhysiometer III:
Staining of hybridoma cells in the cell trap to demonstrate toxin delivery.





NanoPhysiometer: Single cell oxygen consumption



Oxygen measurements of single cells in the NanoPhysiometer cell trap. Pulse voltametry was used to monitor oxygen of a single hybridoma cell in a (15 μm)^3 sensing volume The measurement was repeated five times and mean O_2 profiles were plotted for sensing volumes with and without a cell present.

Oxygen Consumption Rate Approximation for Single Cell:

$I = 2\pi nFADC/\{w \ln(64Dt/w^2)\}$

Electrochemical Methods 2nd Edition, pg. 175

n = 4 $D = 3 \times 10^{-5}$ cm^2/s

l= 15 μm t = 20 sec

w = 5 μm ΔI= 1.4 nA

then **ΔC = 154 μM**

Rate Determination:

Rate = ΔC * Volume / Time (40 sec)

Time = 20 sec before run + 20 sec run

Rate = 4.6 e-11 mmole/cell/hour

= 0.5 fM/cell/hour

NanoPhysiometer: High-Bandwidth, Single-Cell Monitoring

- Detect fast, direct response rather than slow secondary responses
- Cell can serve as its own control
- Calibration of each cell with standard chemical stimuli prior to agent exposure
- Titrate toxin exposure to avoid desensitization and other suprathreshold effects
- Multiple, parallel assays for statistical reliability

Accomplishments

- **Well Plate Assays – metabolic screening of cell lines**
  - Completed development of a 24-well-plate protocol with which to determine glucose, lactate, and CO2 metabolic rates from each well culture. Demonstrated use of metabolic flux analysis to improve metabolic screening signature analysis.
- **Microphysiometer – expanded capabilities, dose response**
  - Fabricated modified Cytosensor heads for use at Vanderbilt and ECBC/SSBCOM, with sensing capabilities for four analytes, interfaced them to multichannel potentiostats, and demonstrated simultaneous measurements of oxygen, pH, glucose, and lactate in microliter volumes.
  - Generated and analyzed multiparameter metabolic biosignatures for antimycin A, botulism toxin, cholera, cyanide, deoxyglucose, DNP, DFP, NaF, and Ricin.
- **NanoPhysiometer – proof of concept**
  - Fabricated completely at Vanderbilt PDMS BioMEMS devices with planar electrochemicai sensors, nanoliter volumes, and external control hardware
  - Measured the oxygen consumption of a single hybridoma cell

Threat Mechanisms

Detection Schemes

A Deliverable under DARPA/ONR Contract
N66001-01-C-8064

High-Content Toxicology Screening Using Massively Parallel, Multi-Phasic Cellular Biological Activity Detectors MP²-CBAD

Don Berry
Vanderbilt Institute for Integrative Biosystems Research and Education
Owen McGuinness
Department of Molecular Physiology and Biophysics
Vanderbilt University
Nashville, TN

1. Biological Agents a. Biotoxins – Con't

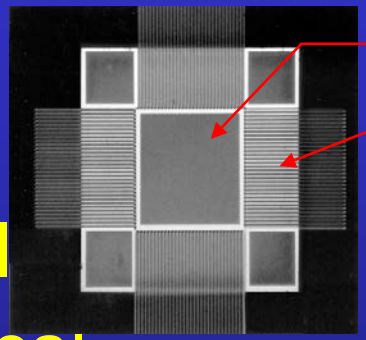
- Microcystins
  - "Cyanobacterial toxins; especially the microcystins (MCYST), hepatocyte damage by inhibiting protein phosphatase 2A, resulting in hyperphosphorylation of cytoskeletal proteins."[McDermott CM, Nho CW, Howard W, Holton B, Toxicon 36: (12) 1981-1996 1998]
  - Since Protein phosphatase 2A (PP2A) plays a central role in essential phosphorylation-dependent signal transduction pathways a test protocol can be generated that exposes cells to microcytins and see a PP2A specific protection. J Pharmacol Exp Ther 1997 Mar;280(3):1152-8
- Cholera Toxin
  - All strains of V cholerae elaborate the same enterotoxin, a protein molecule with a molecular weight of 84,000 daltons. The entire clinical syndrome is caused by the action of the toxin on the intestinal epithelial cell. Cholera toxin causes active secretion of chloride and blocks sodium absorption in the small intestine with the colon being relatively insensitive to the toxin. The large volume of fluid produced in the upper intestine overwhelms the capacity of the lower intestine to absorb. [Biological Warfare agents, Daniel J. Dire, MD. http://www.emedicine.com/emerg/topic853.htm] It activates Gs and thus inhibit glucose uptake. Inhibitors (e.g. H89) of adenyl cyclase will protect against effects of cholera. It activated calcium entry into neuroblastoma cells J Neurosci Res 2002 Sep 1;69(5):669-80
  - Neuroblastoma and hepatocyte cell lines will be very effective and will produce robust inhibition of glucose uptake. In addition cholera toxin must be transported into the cell via a clathrin-independent pathway to exert its effect. Depletion of cholesterol from the cells prior to exposure to the toxin to demonstrate would delineate that internalization is required for the toxin to inhibit glucose uptake.

PROJECTED MILESTONES (NEXT 12 MONTHS)

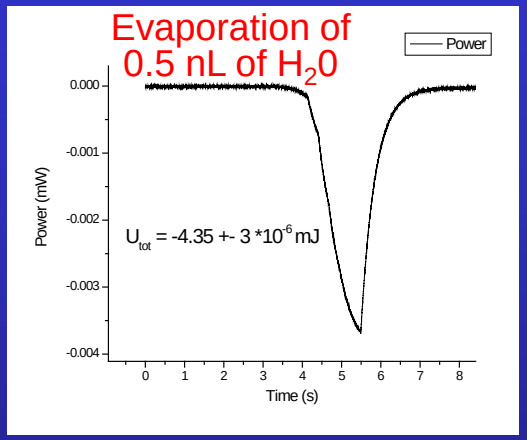
- **Well Plate Assays**
  - Use the acidification, O2, and glucose/lactate/CO2 protocols to optimize test conditions and screen additional cell lines for improved biosignature generation and evaluation.
- **Microphysiometer**
  - Expand dose response database for CBW agents and cell lines at VU and ECBC. Devise data extraction algorithms.
  - Evaluate sensor performance: sensitivity, stability, interaction.
- **NanoPhysiometer**
  - Implement single wells with multiple electrochemical sensors.
  - Build integrated system comprised of on-chip wells, channels, valves, and pumps.

Future Possibilities

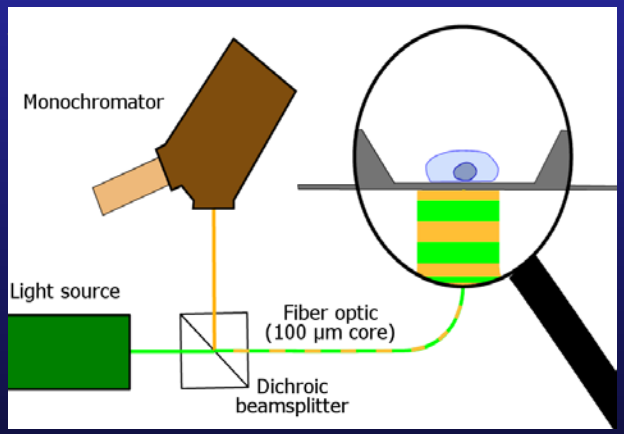
- Additional orthogonal sensors
  - Thermal
  - Optical
  - Electrical
  - Mechanical



Hypres – PicoCalorimeter (bottom view)



- Advanced metabolic and sig analysis



- Instrumented model organisms (c. elegans)

Future Possibilities, Con't

- Predator-prey activity analyzer: bacteria and protozoa in bioremediation
- Natural, genetically engineered, and synthetic ion channels as biosensors
- and other proprietary cellular instrumentation systems

Project Members

- Robert Balcarcel, Chemical Engineering
- Franz Baudenbacher, Physics
- David Cliffel, Chemistry
- Owen McGuinness, Molecular Physiology & Biophysics
- Ales Prokop, Chemical Engineering
- Mark Stremler, Mechanical Engineering
- Roy Thompson, SBCCOM ECBC
- John Wikswo, BME, MPB, Physics

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- Yuansheng Yang, Chemical Engineering
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