Islet Quality Assessment

Clark K. Colton

Department of Chemical Engineering
Massachusetts Institute of Technology
Cambridge, MA

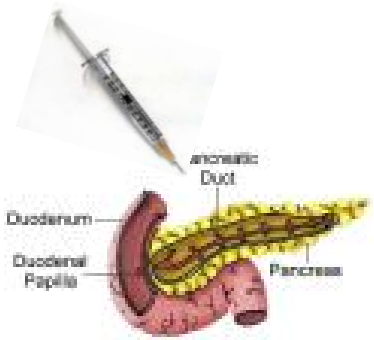
Introductory overview

Quantity and composition of islet preparations
Quantitative membrane integrity measurements
Oxygen consumption rate measurements
- Stirred chamber
  - methods and characteristics
  - prediction of transplantation outcome
- Oxygen biosensor system

Islets Are Damaged During Isolation from Human Pancreas

Enzymatic Digestion and Mechanical Disruption

Distension with Collagenase/Protease solution

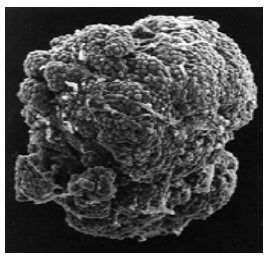


Ischaemic Conditions

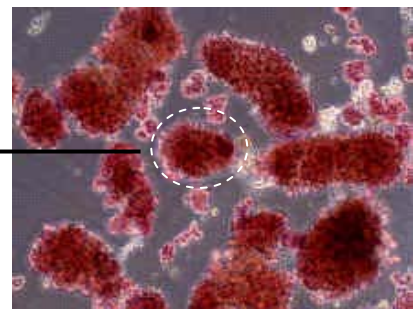
Shaker

Density Gradient Centrifugation → Exocrine Tissue

Islet Preparation



150 µm



1-2% original pancreas volume

What Do We Want To Know?

For a given islet preparation: What is the “potency” or “dose?”
Can we predict transplantation outcome?

Goals for Islet Quality Assessment

Quantity

How much tissue is there?

- Volume
- Number of cells

What is the tissue composition?

- Islet – βcells, other
- Exocrine– acinar, duct

Function

What is the insulin secretory capacity?

Viability

For (1) total tissue and (2) islets

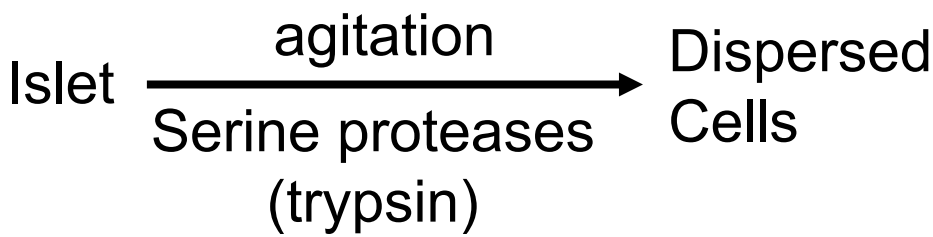
- How much is viable?
- What fraction is viable?

What does viability mean, anyway?

- Dead
- Live
- Live now, dead later because of irreversible commitment to the cell death process

Why Are Islet Preparations So Difficult To Characterize?

1. Islets are cellular aggregates.
   Variety of shapes and sizes
   Visual size estimation is
   - prone to error
   - operator dependent
   - large uncertainty
2. Human preparations have varying amounts of impurities.
   Distinguishing properties of islets/exocrine tissue difficult
3. The islet is a moving target.
   Damage occurs during
   - isolation
   - culture
   - shipment
4. Many techniques for cells are inapplicable to islets because the islets cannot be usefully dissociated into cells.



- Cells are damaged: anoikis
- Cells are lost
- Recovered cells are likely not representative of original islet

What Tools Are Available?

- Safety
- Identity
- Quantity of tissue
  - Volume
  - Number of Cells
  - Composition
- Viability
  - Membrane Integrity
  - Mitochondrial Function
  - Apoptosis
- Potency
  - Glucose Stimulated Insulin Release
  - Immunodeficient Mouse Transplant
- Other
  - Gene Expression Profiling

Quantity of Tissue

Type of Quantity	Tissue Assayed	Parameter Measured	Method
Volume	Islet Preparation	Tissue volume	• Packed cell volume of tissue pellet • Ultrasound scattering
		Islet volume	• Insulin content • Dithizone (DTZ) staining Visual counting / Image analysis } Enumeration of islet equivalents (IEQ)
Number of Cells	Islet Preparation	Total DNA	• DNA content
		Total intact cell nuclei	• Nuclei counting
Cell Composition	Islet Preparation	Volume fraction islets	• DTZ staining • Morphology (light microscopy)
		Individual cell types	• Ultrastructural analysis (electron microscopy)
	Dispersed Cells	Individual cell types	• Differential staining (laser scanning cytometry)

Viability of Tissue

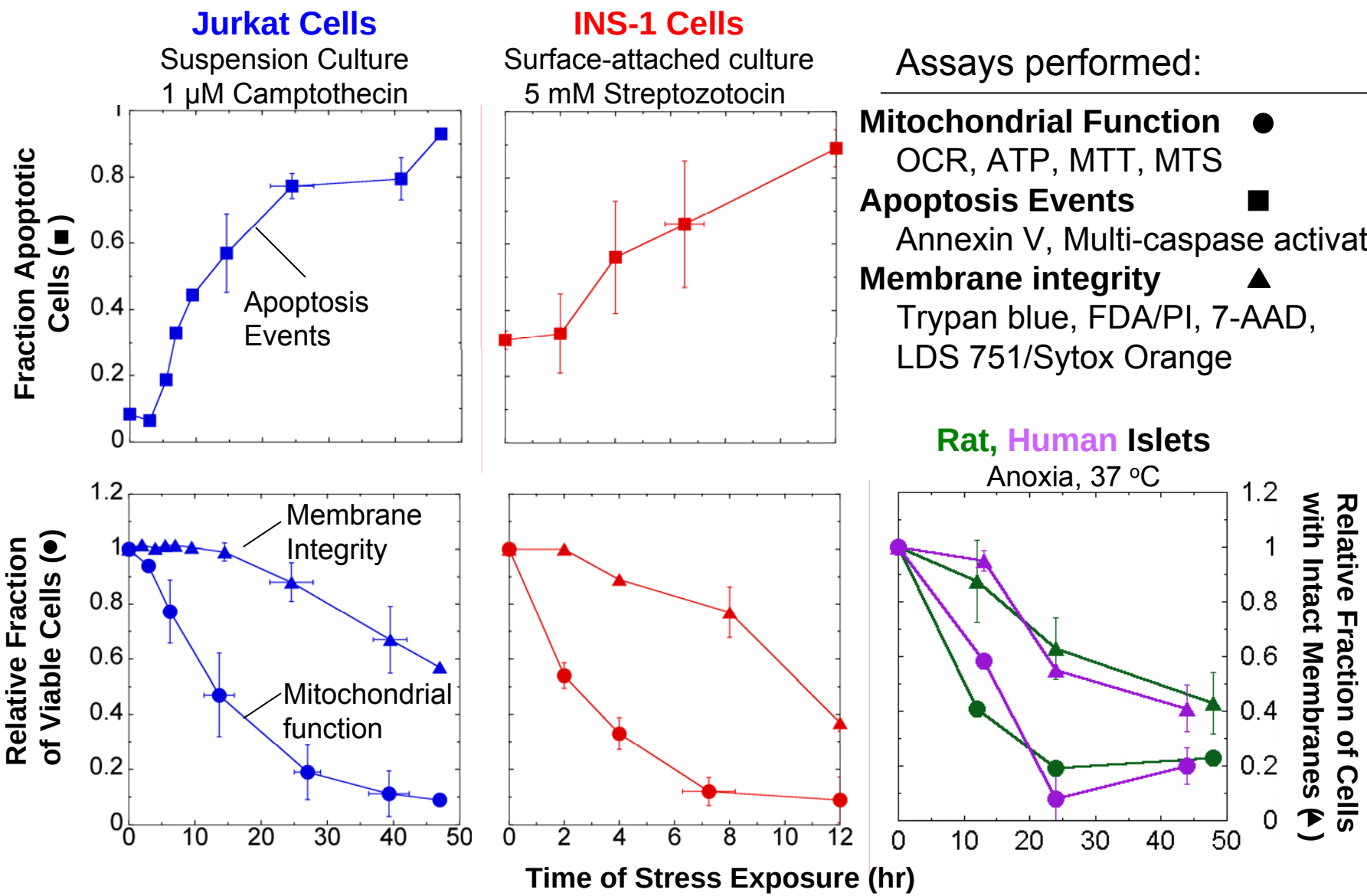
Type of Assay	Tissue Assayed	Method
Cell Membrane Integrity	Islet Preparation	**Live/Dead (Membrane Permeable)** Fluorescein Diacetate (FDA)/Propidium Iodide (PI) SYTO 13/Ethidium Bromide (EB) **All/Dead** LDS 751/Sytox Orange **Dead** Trypan Blue Quantitative assay via Nuclei Counting- 7- AAD
Mitochondrial Function	Islet Preparation	Redox state of the cell-Tetrazolium salts MTT, MTS Oxidative phosphorylation-Oxygen consumption rate Energetic State-[ATP], [ATP]/[ADP], ATP production rate
	Dispersed Cells	Mitochondrion membrane potential (MMP)-Fluorescent dyes JC-1, TMRE (Flow Cytometry)
Apoptotic Events	Islet Preparation	Magic angle spinning 1H-NMR spectroscopy
	Disrupted Cells	Early: Signaling pathway – Caspase activation Late: Nucleosome DNA fragmentation
	Fixed Tissue or Cells	Phosphatidyl serine translocation – Annexin V DNA fragmentation – TUNEL

Viability of Tissue

Type of Assay	Tissue Assayed	Method

Do These Assays Give Equivalent Results?

Time Dependence of Cell Death and Cell Viability Assays



Assays performed:

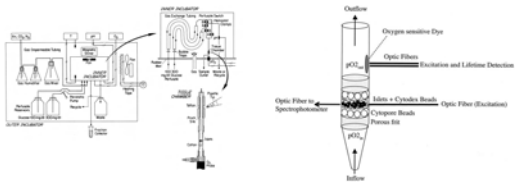
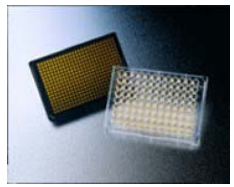
Mitochondrial Function ●
OCR, ATP, MTT, MTS

Apoptosis Events ■
Annexin V, Multi-caspase activation

Membrane integrity ▲
Trypan blue, FDA/PI, 7-AAD, LDS 751/Sytox Orange

Membrane Integrity measurements (7-AAD) lag other measures of cell viability

How Can Oxygen Consumption Rate (OCR) Be Measured?

Hardware	Perfusion Systems	Stagnant Liquid Film	Stirred Tank
Measured Variables	ΔpO_2 across tissue liquid flow rate	Sensor pO_2 beneath cells	$\frac{\Delta pO_2}{\Delta t}$ rate of bulk pO_2 decrease
Source	custom-made	BD Oxygen Biosensor System (BD OBS)	Instech Micro Oxygen Uptake System
			
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Cons	very complex time consuming	measurement is inaccurate Requires mathematical model to calculate OCR	complex

How Can Oxygen Consumption Rate (OCR) be Measured?

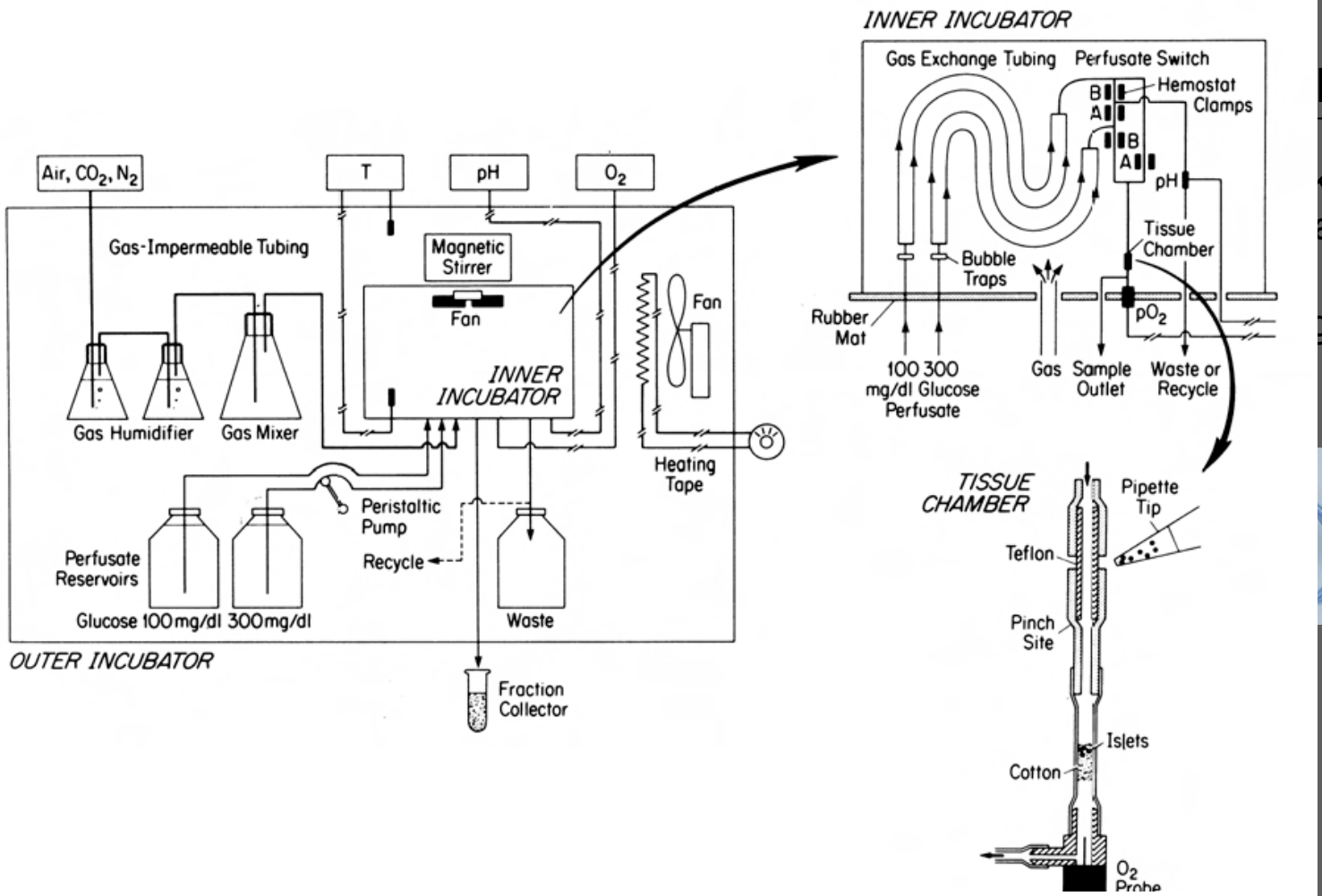
Hardware

Measured Variables

Source

Pros

Cons



Dionne, K.E., et al. A microperifusion system with environmental control for studying insulin secretion by pancreatic tissue, *Biotechnol. Prog.*, **7**, 1991, 359-368

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	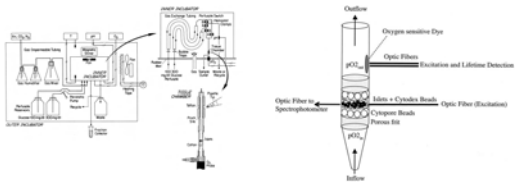	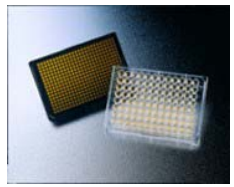	
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	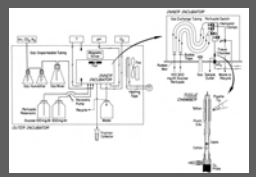
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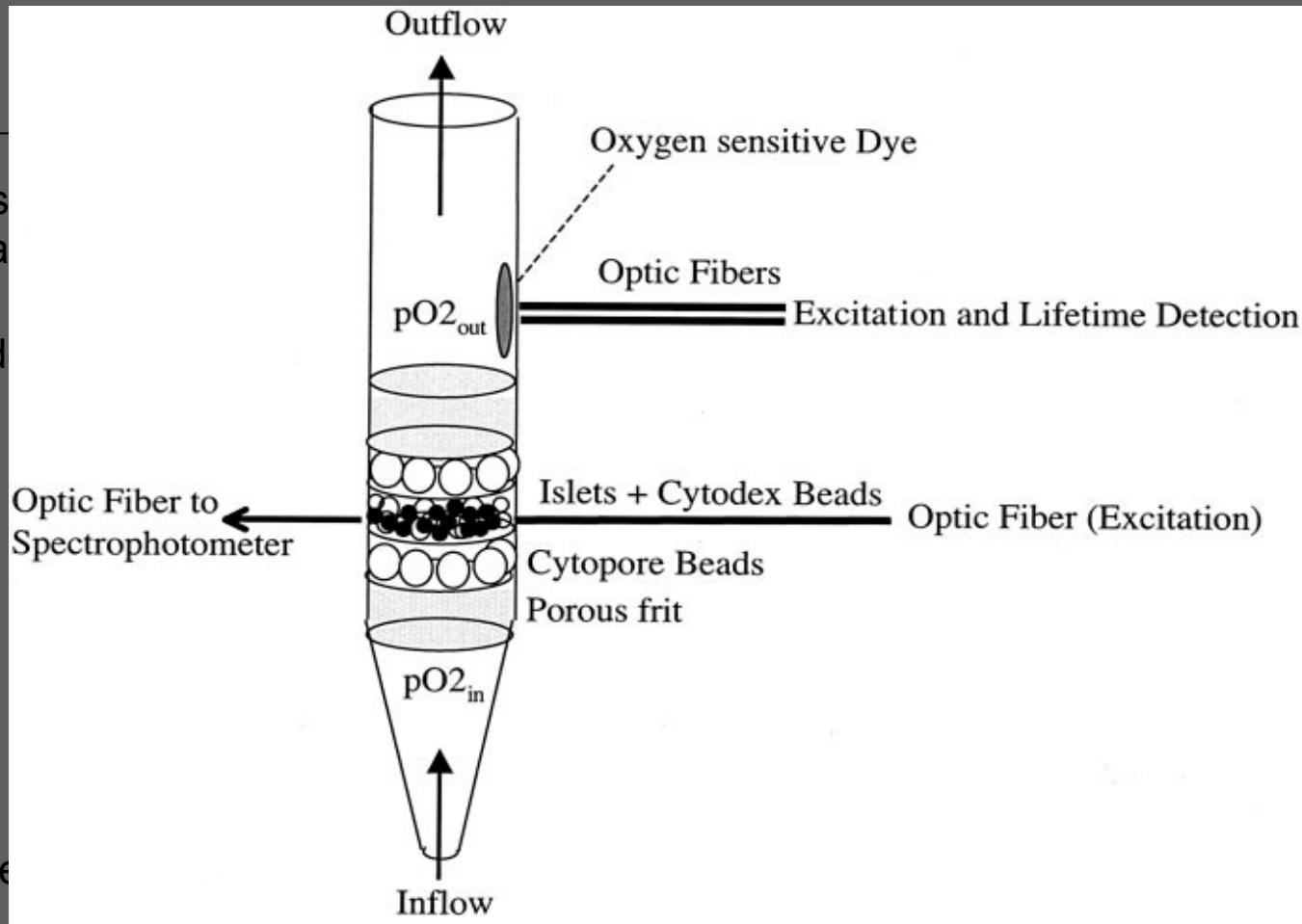

Sweet I.R., et al. Regulation of ATP/ADP in Pancreatic Islets, *Diabetes*, **53**, 2004, 401-409

accuracy is questionable
limited experience

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	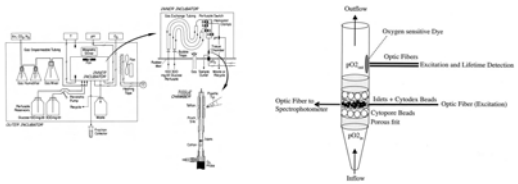	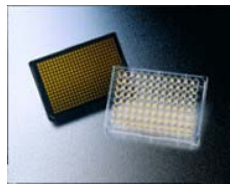	
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How Ca[illegible]?

Hardware	
Measured Variables	
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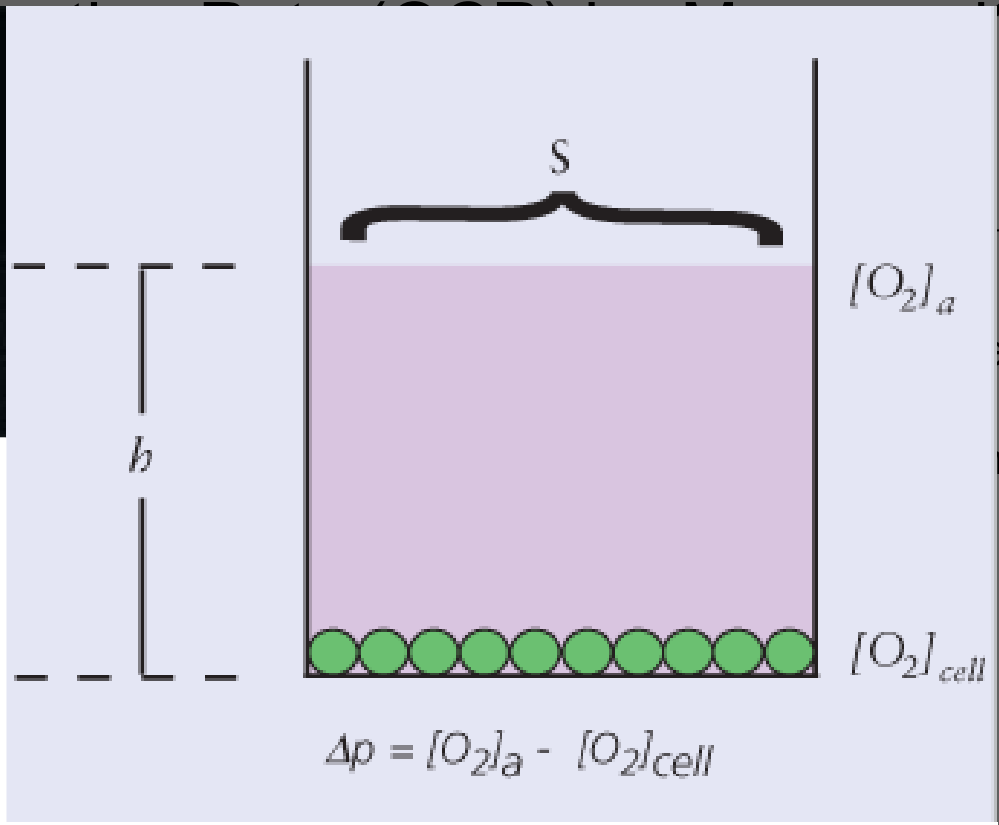

Microplate with oxygen sensitive fluorophor immobilized at the bottom.

Cells or islets are placed within the microplate, settle to the bottom, and consume oxygen

Oxygen partial pressure in the sensor decreases and can be used to estimate the OCR

Guarino, R.D., et al. Method for determining oxygen consumption rates of static cultures from microplate measurements of pericellular dissolved oxygen concentration, *Biotechnol. Bioeng.*, **86**(7), 2004, 775-787

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	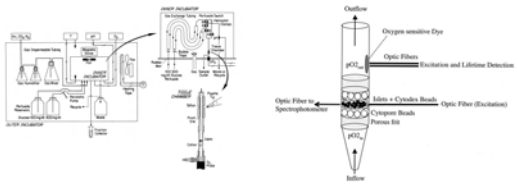	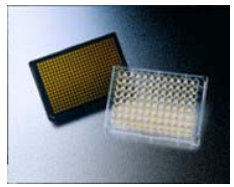	
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How C ed?

Hardware k

Measured Variables

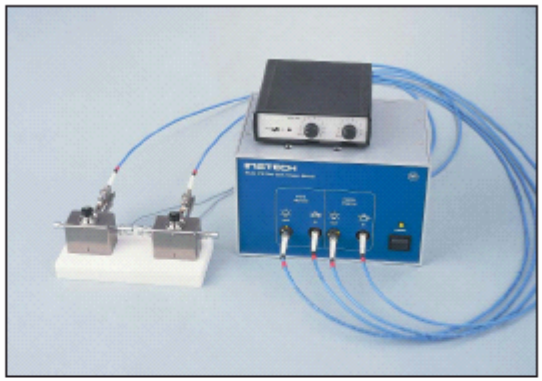
Source

Pros

Cons

Micro Oxygen Uptake System

The Instech FOSYS210T was designed specifically to determine oxygen consumption rates (OCR) of cell suspensions or organelles as well as enzyme reactions that consume or evolve oxygen in samples as low as 200µL. This system was designed by Instech in collaboration with Drs. Kleanthes Papas and Clark Colton of the Department of Chemical Engineering at MIT.



Oxygen tension in the sample fluid is sensed fluorometrically using a fiber optic titanium needle probe coated at the tip with a captured fluorescent dye. Fluorescence levels are detected using the model 210 two-channel monitor which incorporates the excitation light sources, miniature spectrometers and a high-speed A/D converter. Titanium is used for the chamber body and probe since it is inert and will not introduce drifts due to material oxidation while providing for rapid thermal equilibrium of solutions. A standard user-provided PC with USB port and the included software control all monitor functions. Data is displayed on the screen and can be logged to disk and recalled for analysis.

The part number FO/SYS210T specifies a complete system including:

1 Two channel fiber optic oxygen monitor
2 0.062" OD titanium fiber optic oxygen probes
2 400µ bifurcated fiber bundles with couplers
1 Dual water jacketed micro chamber block
2 250µL titanium chamber cups
2 Glass plug/valves for 250µL chambers
2 Acrylic center-fill plugs for 250µL chambers
1 Two channel speed controller
2 Low speed motor-magnet stirring assemblies
2 Glass coated 5mm stir bars
1 Probe seal kit (25 pcs)
1 Thermocouple thermometer

The only part not included in the FO/SYS210T system is the Haake circulating water bath, FO/C70B, described below:

Part No.	Description	Unit
FO/SYS210T	Dual micro oxygen uptake system	ea

Titanium Fiber Optic Probes

The oxygen sensing probe is constructed of rugged, .062" diameter titanium tube with a 600 micron central fiber that has been coated with a fluorescent dye instilled in a sol-gel coating at the tip. The tip is then overcoated with a thin layer of oxygen permeable black silicone. This prevents ambient light interference with the measurement. Because of the small size of these probes, it is possible to achieve low chamber volumes.

Unlike polarographic probes, these probes require neither membranes nor electrolytes, making for less maintenance and longer times between calibration. Once the probe tip comes into equilibrium with the surrounding oxygen concentration, no further oxygen utilization occurs. Since the probe does not consume oxygen, there will be no errors due to probe uptake and no stirring artifacts. The probes are capable of gaseous measurements as well but should be calibrated in solution when making dissolved oxygen measurements. Only aqueous solutions should be used with these probes to prevent leaching out of the dye.

Part No.	Description	Unit
FO/P062T	.062" titanium fiber optic probe with silicone overcoat	ea
FO/FC400	400µ bifurcated fiber bundle with coupler	ea

Instech Laboratories, Inc. • 5209 Militia Hill Road, Plymouth Meeting, PA 19462-1216, USA
800-443-4227 • 610-941-0132 • 610-941-0134 fax • www.instechlabs.com

INSTECH

Micro Oxygen Uptake System

Built into the chamber block is a miniature magnetic stirring system that employs high strength, neodymium iron boron magnet to ensure constant coupling of the tiny stir bar even at highest speeds. The drive motor is geared down for accurate slow speed stirring when used with fragile cells. The Instech 2060 speed controller provides accurate, reproducible rotational speeds.

The Haake DC10-B3 circulating water bath provides temperature control when attached to the ports on the chamber block. A thermocouple thermometer is recommended to verify chamber block temperature.

Part No.	Description	Unit
FO/CS250	Dual 250µL titanium chamber system	ea
FO/C70B	Haake DC10-B3 circulating water bath	ea
FO/CTC	Thermocouple thermometer	ea

Chamber System Replacement Parts

Part No.	Description	Unit
FO/CP2500	Glass plug/valve for 250µL chamber	ea
FO/CP250P	Acrylic center-fill plug for 250µL chamber	ea
FO/CC250	Titanium 250µL chamber	ea
FO/CP500P	Acrylic center-fill plug for 500µL chamber	ea
FO/CC500	Titanium 500µL chamber	ea
FO/CP1000P	Acrylic center-fill plug for 1000µL chamber	ea
FO/CC1000	Titanium 1000µL chamber	ea
FO/CM250K	Professional kit	pkg of 25
FO/C2X5	Glass covered 5mm stir bars	pkg of 3
FO/C2040	Two channel stirring speed controller	ea

System Specifications

210 Monitor	
Channels	2
Excitation wavelength	450nm
Communication	USB (cable included)
Power source	12VDC 800Ma wall mounted adapter
Power consumption	2.4W
Dimensions	8.3/8"W x 5 1/4"H x 6"D
Weight	3.0 lbs

FO/CS250 Chamber System	
No. of Chambers	2
Chamber material	Titanium
Chamber volume	250µL included (500µL and 1000µL available)
Chamber plugs	Glass plug/valve or acrylic center-fill
Chamber block material	Nickel-Teflon coated aluminum
Water bath ports	Fit 5/16" ID Tygon
Stirring	Integral motor/magnet assembly
Stirring speed control	Model 2060 dual speed controller

FO/P062T Fiber Optic Probes	
Material of needle	Titanium
Fiber	Coated 600µ
Fluorescence peak	600nm
OD of needle section	0.0625"
Length of needle section	1.25"
Connection	SMA
Dynamic range	0 - 48.7 ppm (0 - 768 mmHg)
Response time	30-50 sec
Resolution @ 0 ppm	0.005 ppm
Resolution @ 8.5 ppm	0.02 ppm
Resolution @ 40 ppm	0.2 ppm
Drift	<0.02 ppm per day

FO/C70B Circulating Water Bath	
Accuracy	±0.02°C
Volume	3L
Range (no cooling)	35 - 100°C
Range (tap water cooling)	20 - 100°C
Weight	16 lbs

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Commercially Available from Instech Labs
http://www.instechlabs.com/Oxygen/

research tool
not for routine use

accuracy is questionable
limited experience

sive

Summary: Where Are We?

Quantity

Volume

- Packed Cell Volume
- Insulin Content
- Dithizone staining
  - IEQ enumeration
- Ultrasound Scattering

Number of Cells

- DNA
- Nuclei Counting

Cell Composition

- Dithizone Staining
- Morphology (Light Microscopy)
- Ultrastructural Analysis (Electron Microscopy)
- Differential Staining (Laser Scanning Cytometry)

Viability

Mitochondrial Function

Intact Islets:	Redox state of the cell – Tetrazolium salts MTT, MTS
	Oxidative phosphorylation – oxygen consumption rate (OCR)
	Energetic State – [ATP], [ATP]/[ADP], ATP production rate
Single Cells:	Mitochondrial membrane potential (MMP) – Fluorescent dyes JC-1, TMRE

Apoptotic Events

Intact Islets

- Magic angle spinning ^{1}H-NMR spectroscopy

Disrupted Islets

- Early: Signaling pathway – Caspase activation
- Late: Nucleosome DNA fragmentation

Fixed Tissue or Cells

- Phosphatidyl serine translocation – Annexin V
- DNA fragmentation – TUNEL

Cell Membrane Integrity (Intact Islets)

Live/Dead (Membrane Permeable)

- Fluorescein Diacetate (FDA)/Propidium Iodide (PI)
- SYTO 13/Ethidium Bromide (EB)

All/Dead - LDS 751/Sytox Orange

Dead - Trypan Blue

Quantitative assay via Nuclei Counting

- 7- aminoactinomycin D (7AAD)

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Quantity of Tissue

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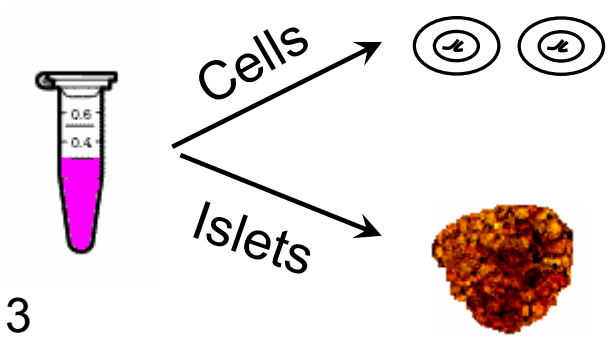
Number of Cells	Islet Preparation	Total DNA	• DNA content
		Total intact cell nuclei	• Nuclei counting

Nuclei Counting Protocol



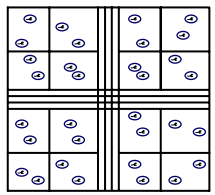
Citric Acid
Surfactant

Vortex Mixing (Cells)
Shearing through
needle (Islets)

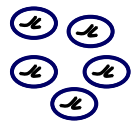
Liberated
Nuclei

10^3 nuclei x 3
Time (min)

70

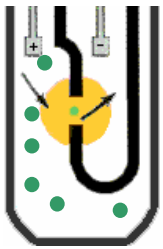


Visual Counting
Hemacytometer



Crystal Violet

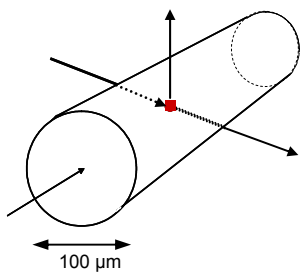
16



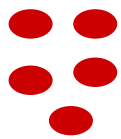
Aperture Resistance
Coulter Multisizer II

40 % (v/v) glycerol
in Isoton II

11

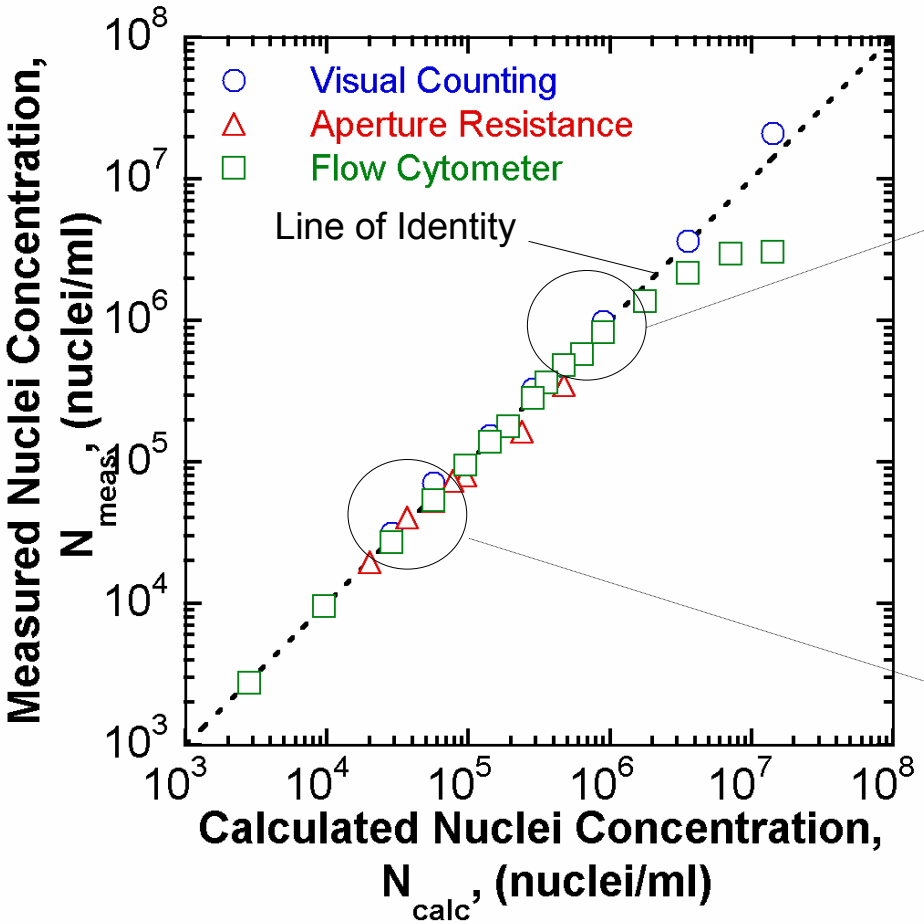


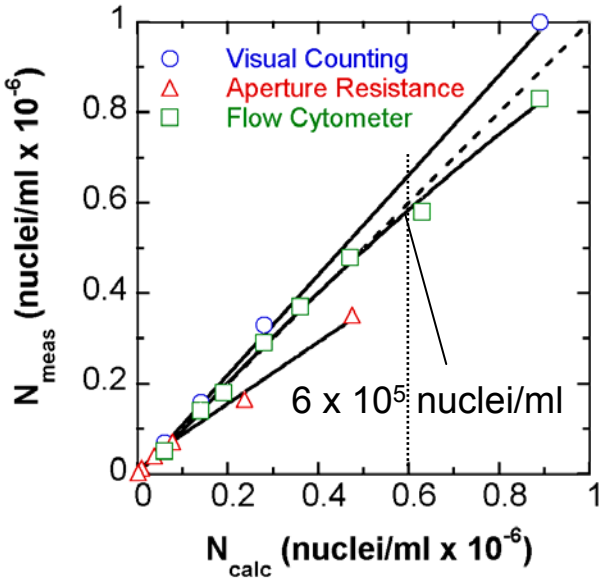
Flow Cytometer
Guava PCA

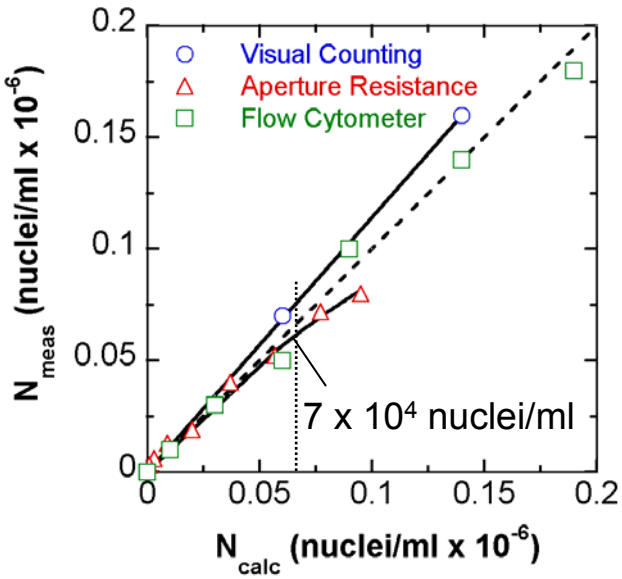


7-AAD

Measured versus Calculated Nuclei Concentration

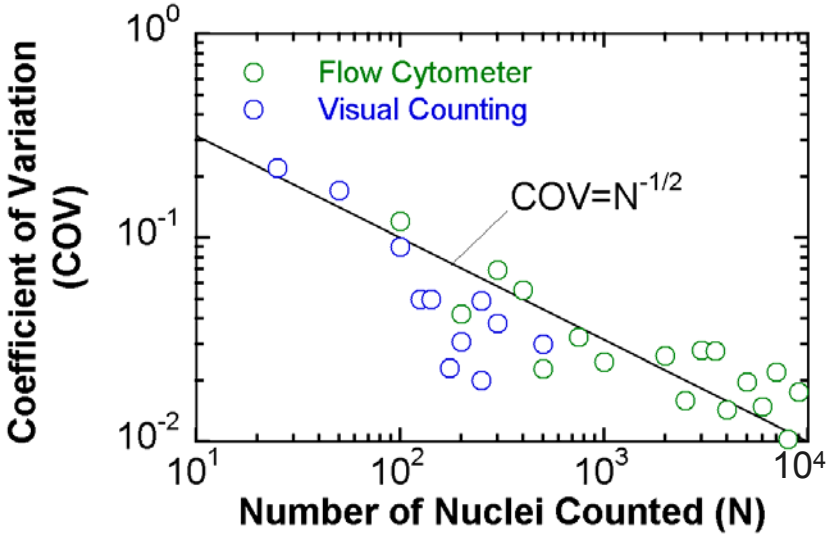


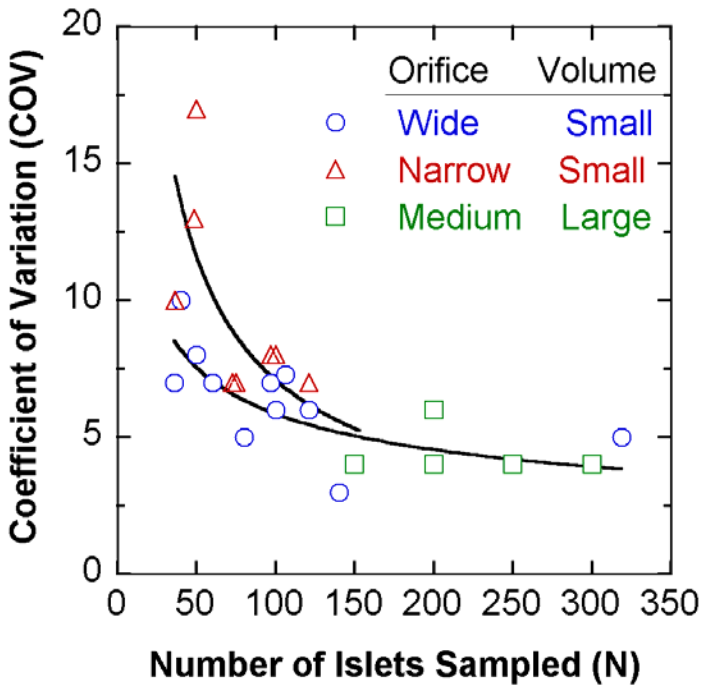




Visual Counting gives slightly high estimate because some fragments are included along with nuclei

Precision of Measurements





- Visual counting and flow cytometry follow approximately Poisson statistics

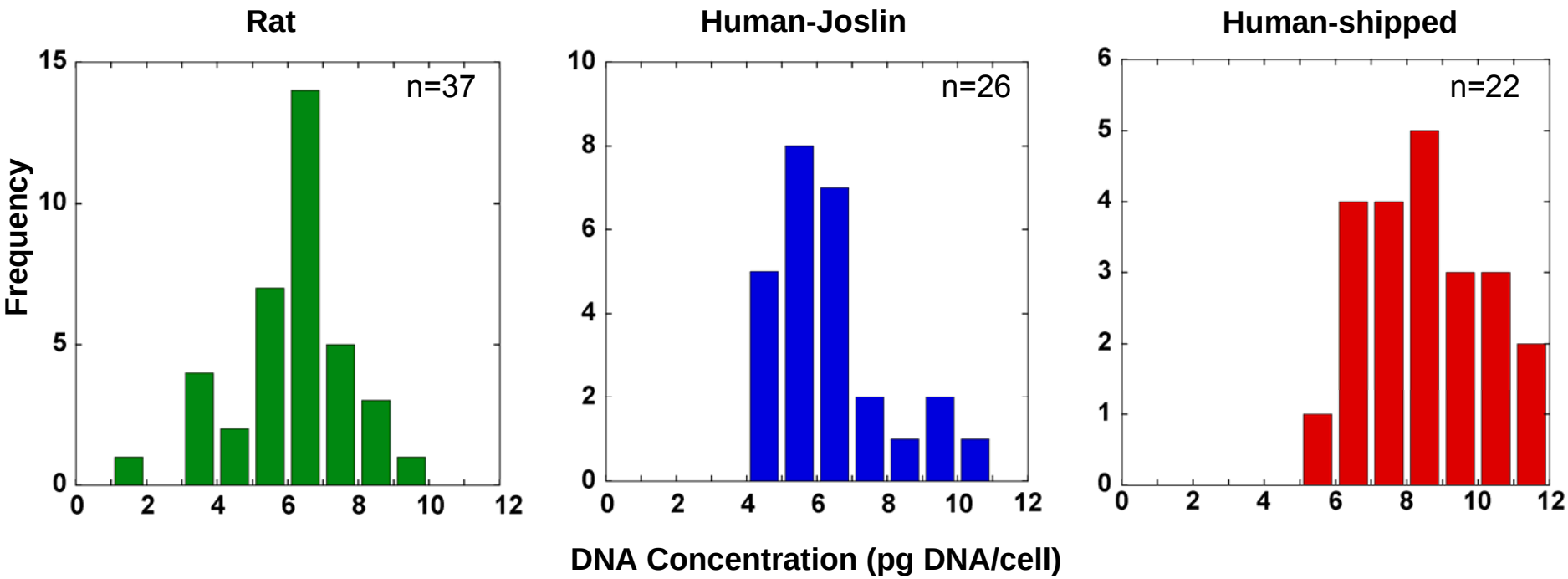
  For cells, $N=10^3$, COV ≈ 3 %

- Precision with islets depends on number of islets sampled and pipette tip used

  For 125+ IEQ, COV ≤ 6 %

DNA Content* Per Cell Based on Nuclei Counting

Islet Sources: Rat and fresh human islets from Joslin Diabetes Center
Shipped human islets from other centers



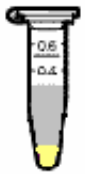
	Rat	Human-Joslin	Human-shipped
Mean ± SD	6.5 ± 1.9	6.9 ± 2.3	8.5 ± 2.3

*DNA data obtained using CyQUANT dye. Different results obtained using PicoGreen.

Quantity of Tissue

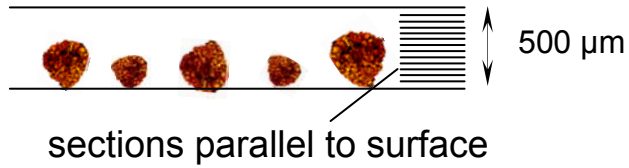
Type of Quantity	Tissue Assayed	Parameter Measured	Method
Cell Composition	Islet Preparation	Volume fraction islets	• DTZ staining • Morphology (light microscopy)
		Individual cell types	• Ultrastructural analysis (electron microscopy)
	Dispersed Cells	Individual cell types	• Differential staining (laser scanning cytometry)

Cell Composition of Human Islet Preparations

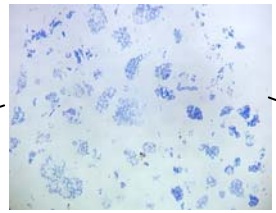


Fix, dehydrate, clear, embed, cure, trim

3 or 4 days



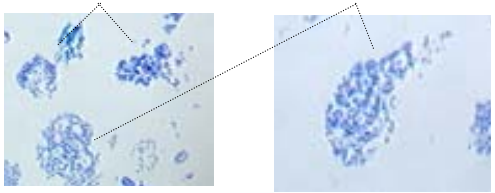
1 µm section



500-800 cells analyzed

Light Microscopy

Non-islet | Islet



10 min

Stereological point counting

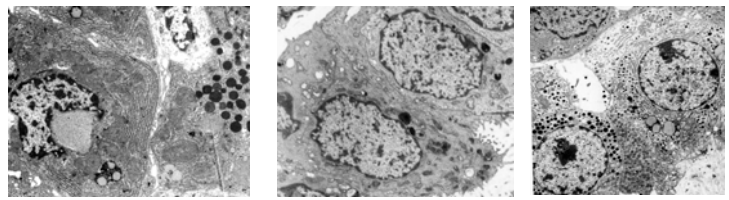
↓

Volume fraction islets, f_L

2 hr

Electron Microscopy

Acinar | Duct | Islet



Individual cell counting

↓

Number fraction islets, f_E

Preparation	Fraction Islets (%) Light f_L	EM f_E	DTZ f_{DTZ}	$\frac{\overline{f}_{L+E}}{\overline{f}_{DTZ}}$	N_{Total} (10^6 cells)	N_{Islets} (10^6 cells)	IEQ Nuclei Counting	IEQ Conventional Method*
1	0.60 ± 0.10	0.49	0.85	0.64	-	-	-	-
2	0.56 ± 0.01	0.62	0.90	0.66	-	-	-	-
3	0.66 ± 0	0.68	0.80	0.84	-	-	-	-
4	0.86 ± 0	-	0.95	0.91	10.8	9.3	47,000	100,000
5	0.64 ± 0.01	-	0.80	0.80	6.4	4.1	21,000	55,000

$$N_{Islets} = f_L \cdot N_{Total}$$

$$IEQ = \frac{N_{Islets}}{2000}$$

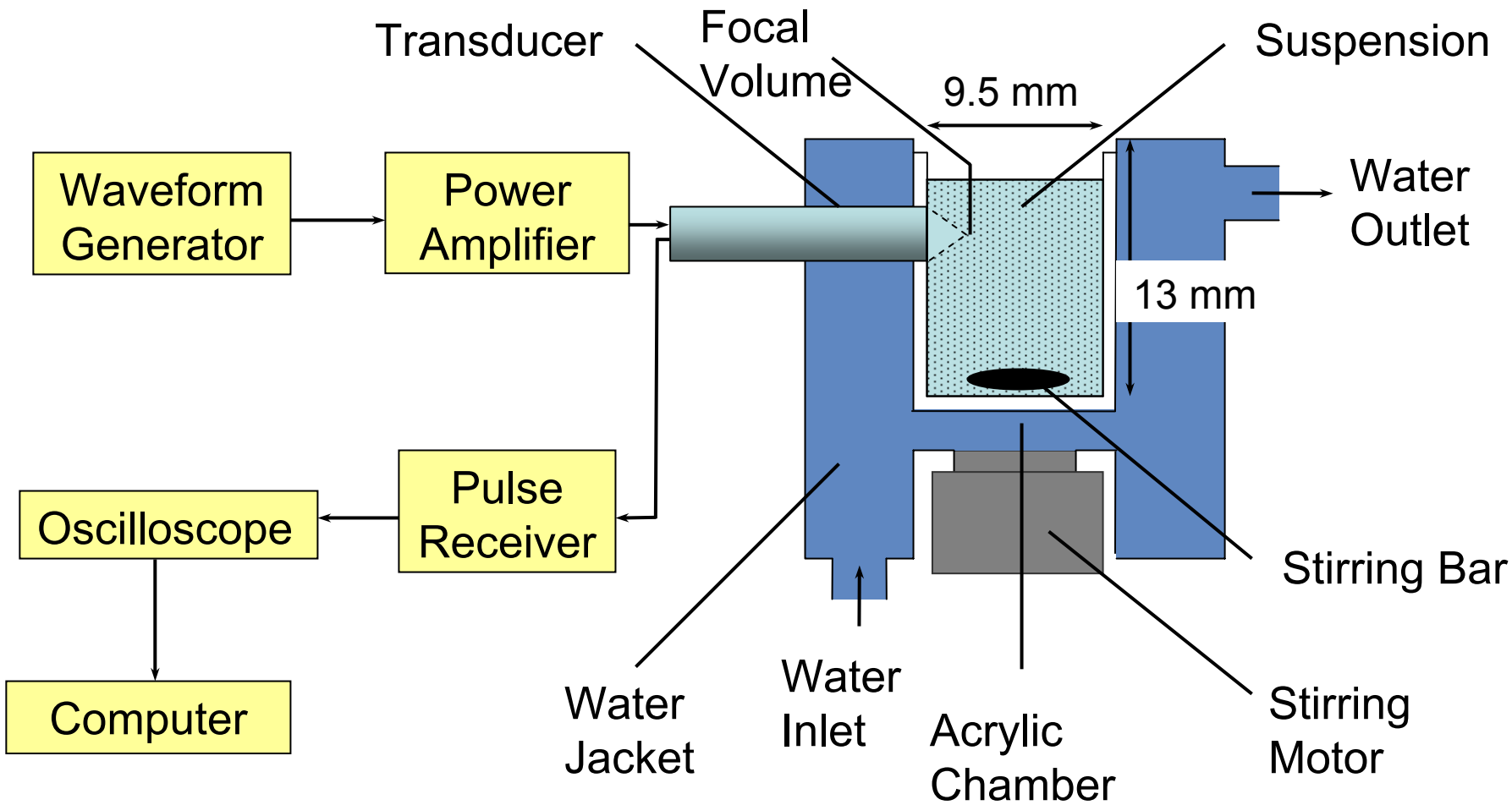
* Reported by the isolation center

Quantity of Tissue

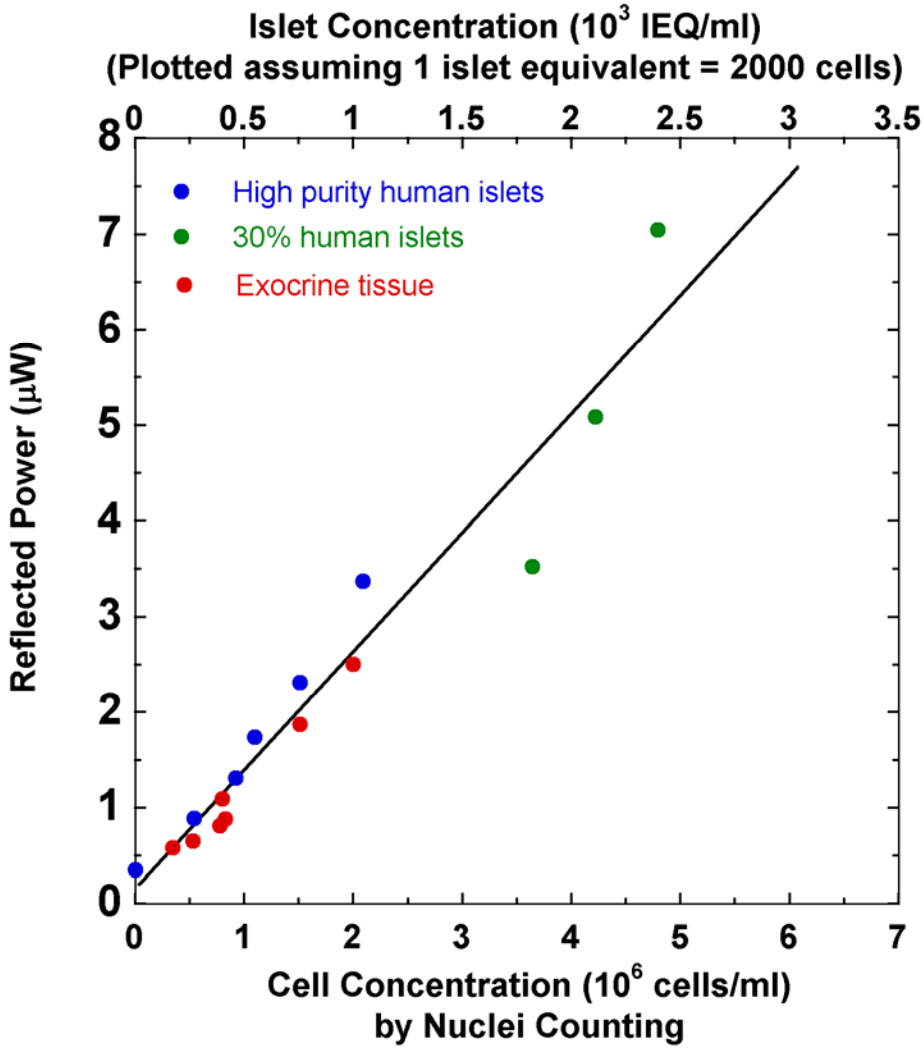
Type of Quantity	Tissue Assayed	Parameter Measured	Method
Volume	Islet Preparation	Tissue volume	• Packed cell volume of tissue pellet • Ultrasound scattering
		Islet volume	• Insulin content • Dithizone (DTZ) staining Visual counting, Image analysis } Enumeration of islet equivalents (IEQ)

Ultrasound Pulsed Doppler (USPD) Measurement of Particle Concentration

System Arrangement and Test Chamber



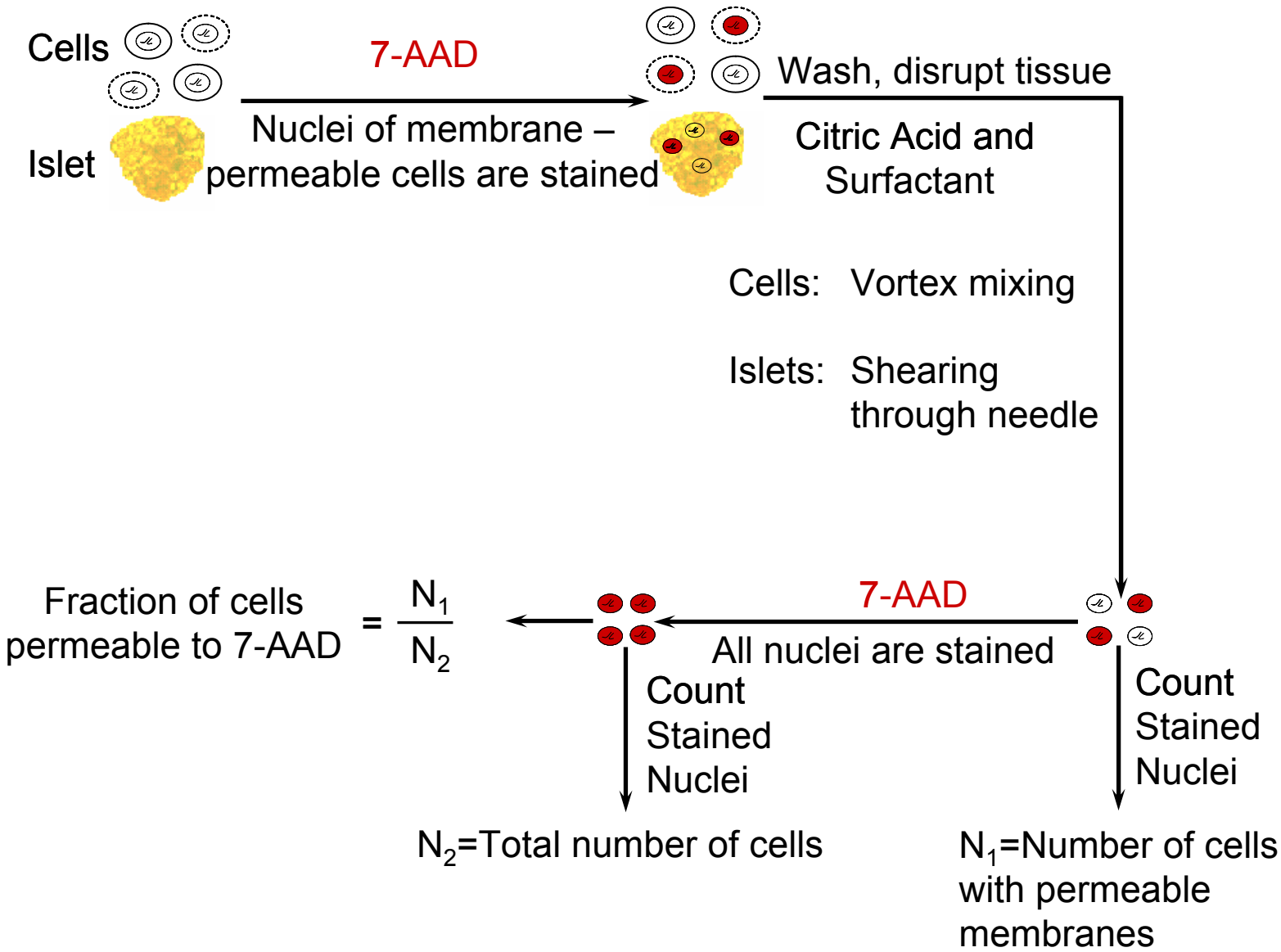
USPD Reflected Power versus Tissue Concentration



Viability of Tissue

Type of Assay	Tissue Assayed	Method
Cell Membrane Integrity	Islet Preparation	**Live/Dead (Membrane Permeable)** Fluorescein Diacetate (FDA)/Propidium Iodide (PI) SYTO 13/Ethidium Bromide (EB) **All/Dead** LDS 751/Sytox Orange **Dead** Trypan Blue Quantitative assay via Nuclei Counting- 7-aminoactinomycin D

Quantitative Membrane Integrity Protocol



Procedure for Validating New Test

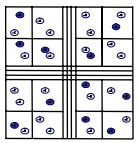
Cells or Islets

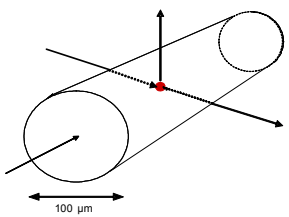
Store on ice → Live Tissue

60 °C, 45 min → Heat-killed Tissue

Mixture Composition (%)

100	75	50	25	0
0	25	50	75	100





Cells Islets

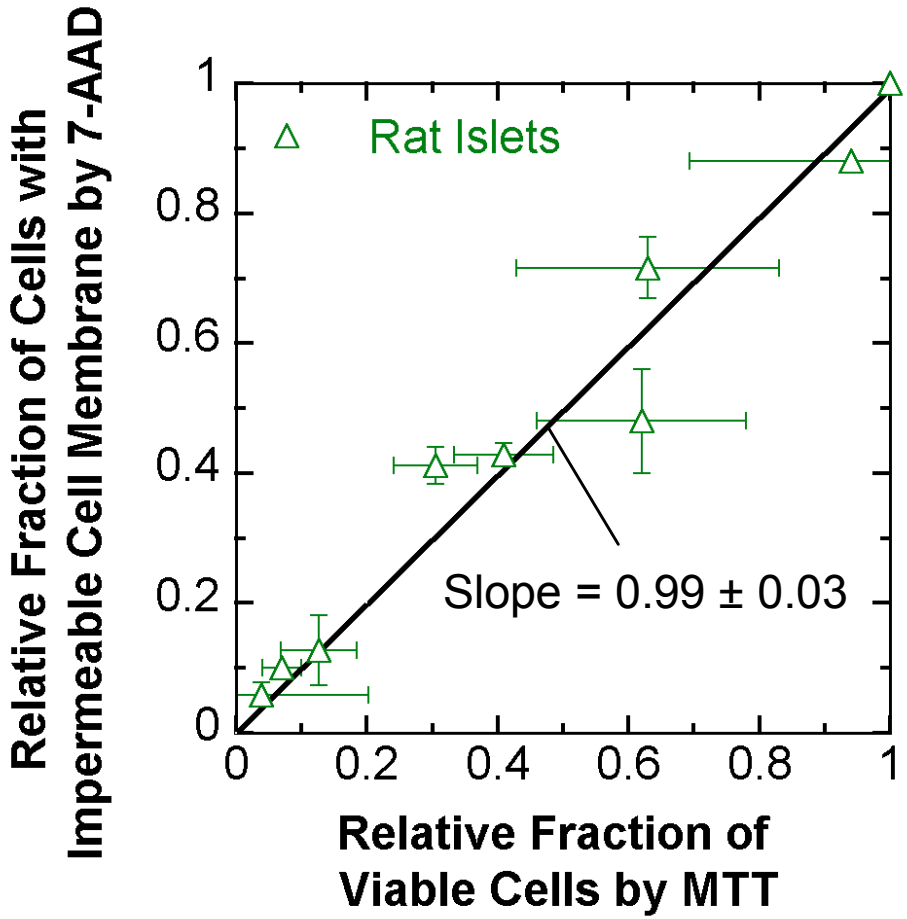
Trypan Blue → **Count stained cells** Hemacytometer

MTT → **Assay viability of cells and intact islets** Plate Reader

7-AAD → **Count stained nuclei** Flow Cytometer (Guava PCA)

Quantitative Membrane Integrity Protocol

Comparison of 7-AAD Sequential Staining with MTT assay with Islets



Nuclei Counting: Conclusions

Nuclei counting provides rapid, accurate, and precise quantitative measurements that can be used advantageously

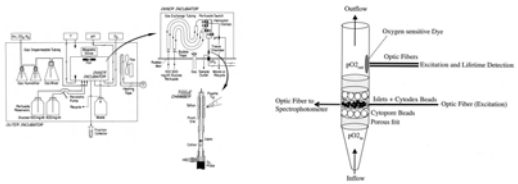
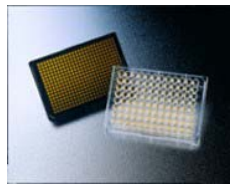
1. Nuclei counting can measure the number of cells in an islet preparation. Combination with microscopic observations (Light and/or EM) gives a reliable, quantitative estimate of the number of islet cells (IEQs) in impure islet preparations.

2. Sequential staining of nuclei with 7-AAD before and after cell disruption, followed by nuclei counting with a flow cytometer, provides an estimate of the fraction of cells that have compromised membrane integrity

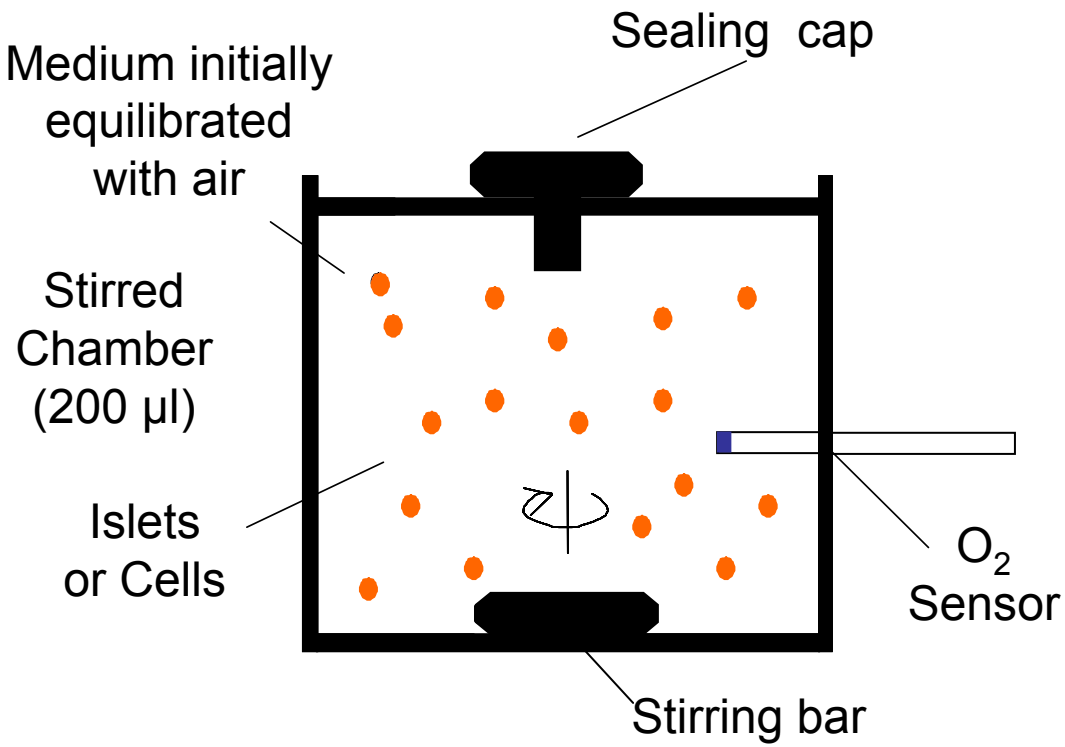
Viability of Tissue

Type of Assay	Tissue Assayed	Method
Mitochondrial Function	Islet Preparation	Redox state of the cell-Tetrazolium salts MTT, MTS Oxidative phosphorylation-Oxygen consumption rate (OCR)
	Dispersed Cells	Energetic State-[ATP], [ATP]/[ADP], ATP production rate

How Can Oxygen Consumption Rate (OCR) Be Measured?

Hardware	Perfusion Systems	Stagnant Liquid Film	Stirred Tank
Measured Variables	ΔpO_2 across tissue liquid flow rate	Sensor pO_2 beneath cells	$\frac{\Delta pO_2}{\Delta t}$ rate of bulk pO_2 decrease
Source	custom-made	BD Oxygen Biosensor System (BD OBS)	Instech Micro Oxygen Uptake System
			
Pros	elegant flexible research tool follow transient dynamics Direct measurement of OCR	simple inexpensive rapid	accurate precise rapid Direct measurement of OCR
Cons	very complex time consuming	measurement is inaccurate Requires mathematical model to calculate OCR	complex

Instech Stirred Chamber for OCR Measurements Schematic Diagram



Water jacketed titanium chamber with fluorescence-quenched O_2 sensor

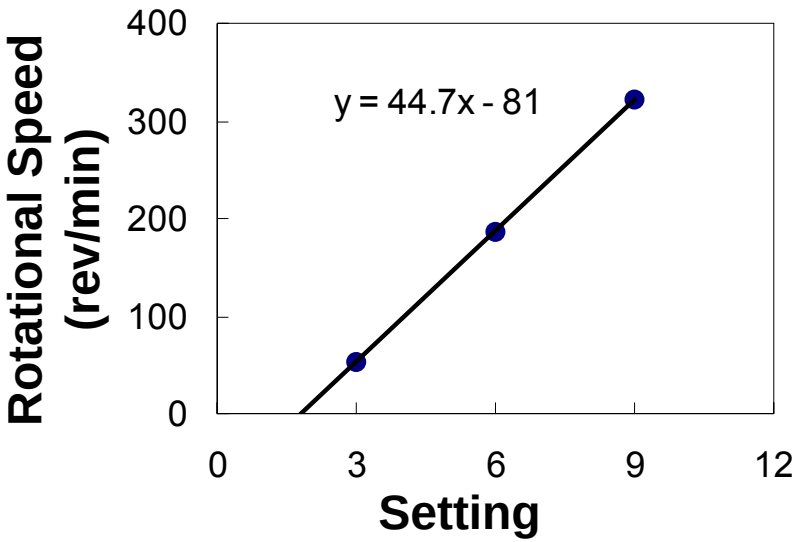
Characteristics of OCR Measuring Chamber

Chamber volume (μl):

MIT	cap 1	200 ± 3, 198 ± 2
	cap 2	177 ± 3, 175 ± 2
Joslin		205 ± 1, 210 ± 3

Stirrer rotational speed:

50 - 300 rpm (setpoint dependent)



Temperature equilibration:

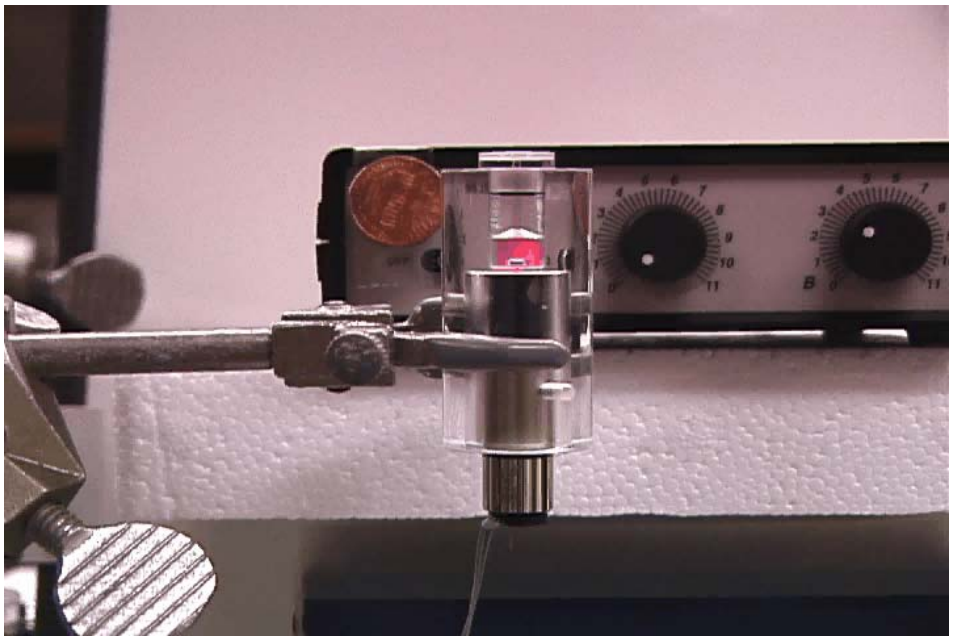
Complete in 15 seconds

O_2 leakage rate: 0-0.2 mmHg/min mmHg (cap dependent)

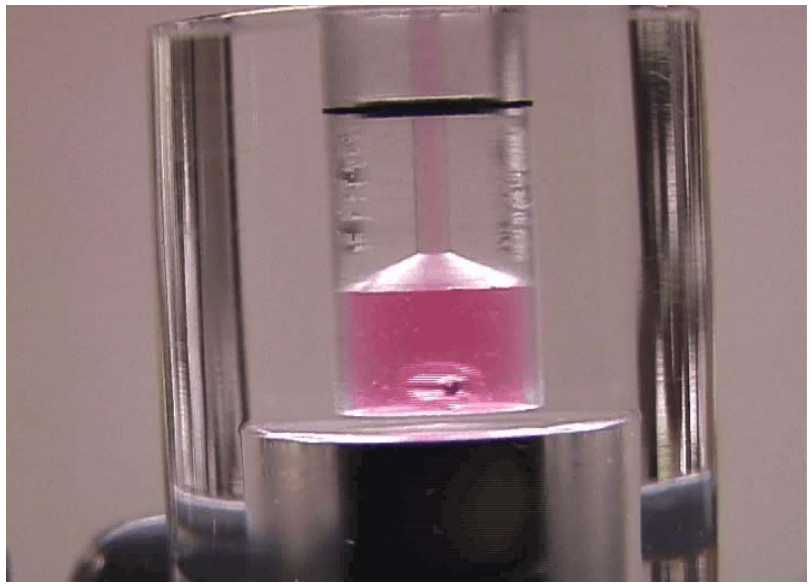
Recovery of tissue after OCR measurement: 1.003 ± 0.043

Sensor Calibration: 0 and 160 mmHg

Flow Visualization in Transparent Model



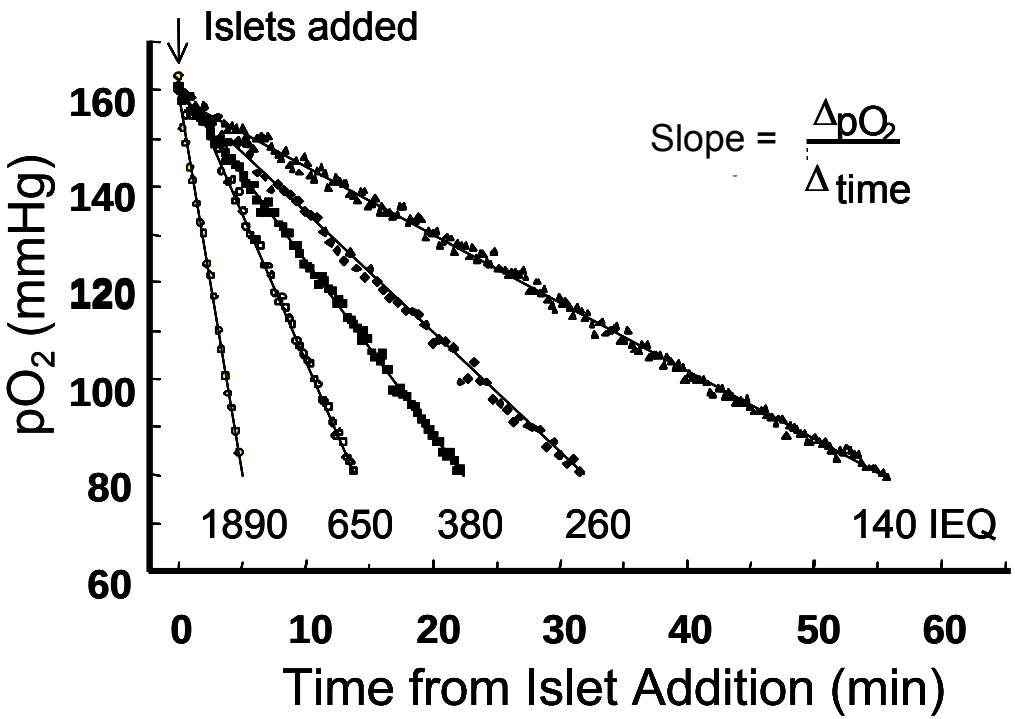
Chamber diameter is about 6 mm
Stirring bar length is about 3 mm



Islets suspension is stirred at the minimum speed to suspend the islets

Measurement of Oxygen Consumption Rate

Actual data fit to straight lines



Calculation of OCR

$$OCR = V_{ch} \cdot \alpha \cdot slope$$

$$\frac{mol}{min} \quad ml \quad \frac{mol}{mmHg \cdot ml} \quad \frac{mmHg}{min}$$

$$\frac{OCR}{cell} = \frac{OCR}{number\ of\ cells}$$

or

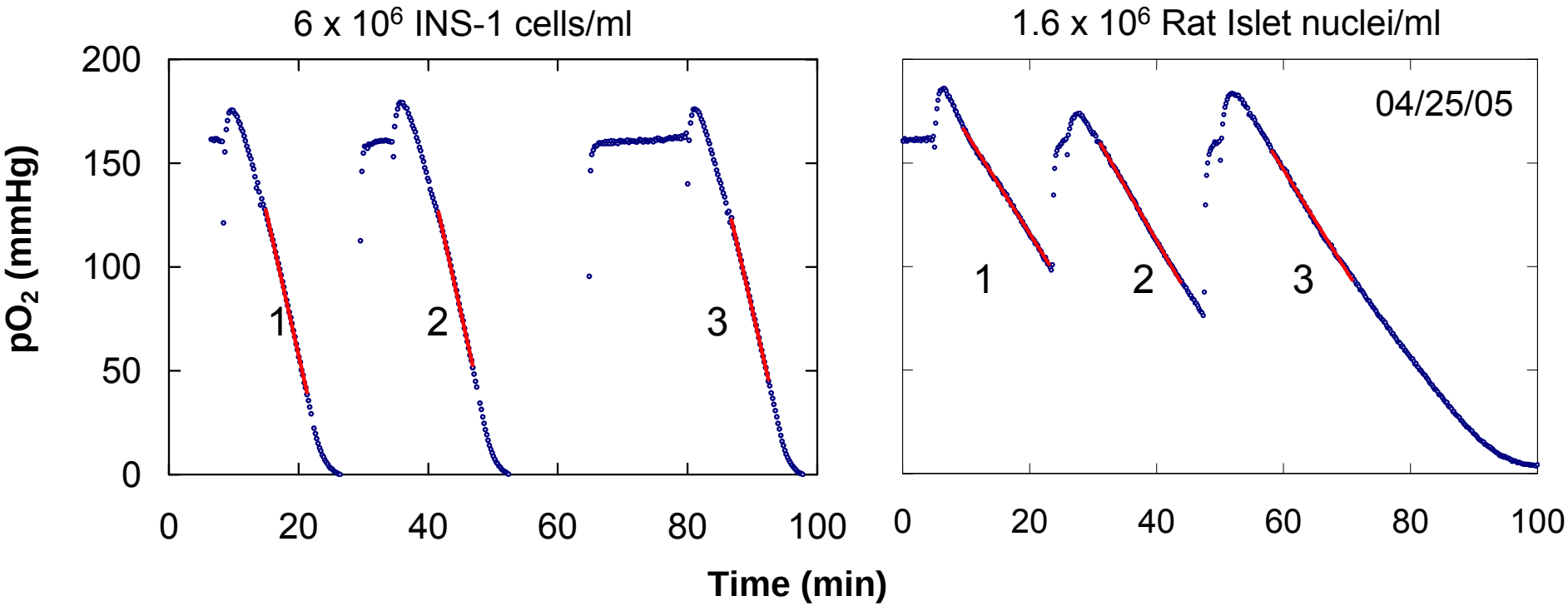
$$\frac{OCR}{cell} = \frac{\alpha \cdot slope}{cell\ concentration}$$

Same for $\frac{OCR}{DNA}$ and $\frac{OCR}{nucleus}$

OCR Chamber Troubleshooting

Problems	Suggestions
Bubble Formation	
Chipped or defective sealing cap	Use undamaged caps (no chipping)
Incomplete filling	Use excess tissue suspension
Low temperature of suspension	Let tissue suspension warm in chamber before sealing
Inadequate Passivation	Passivate
Inadequate Stirring	Check stirring bar is rotating occasionally
Decrease in sensor sensitivity	Recoat sensor every 6 months

Reproducibility: Typical Triplicate Measurements with Fresh Samples



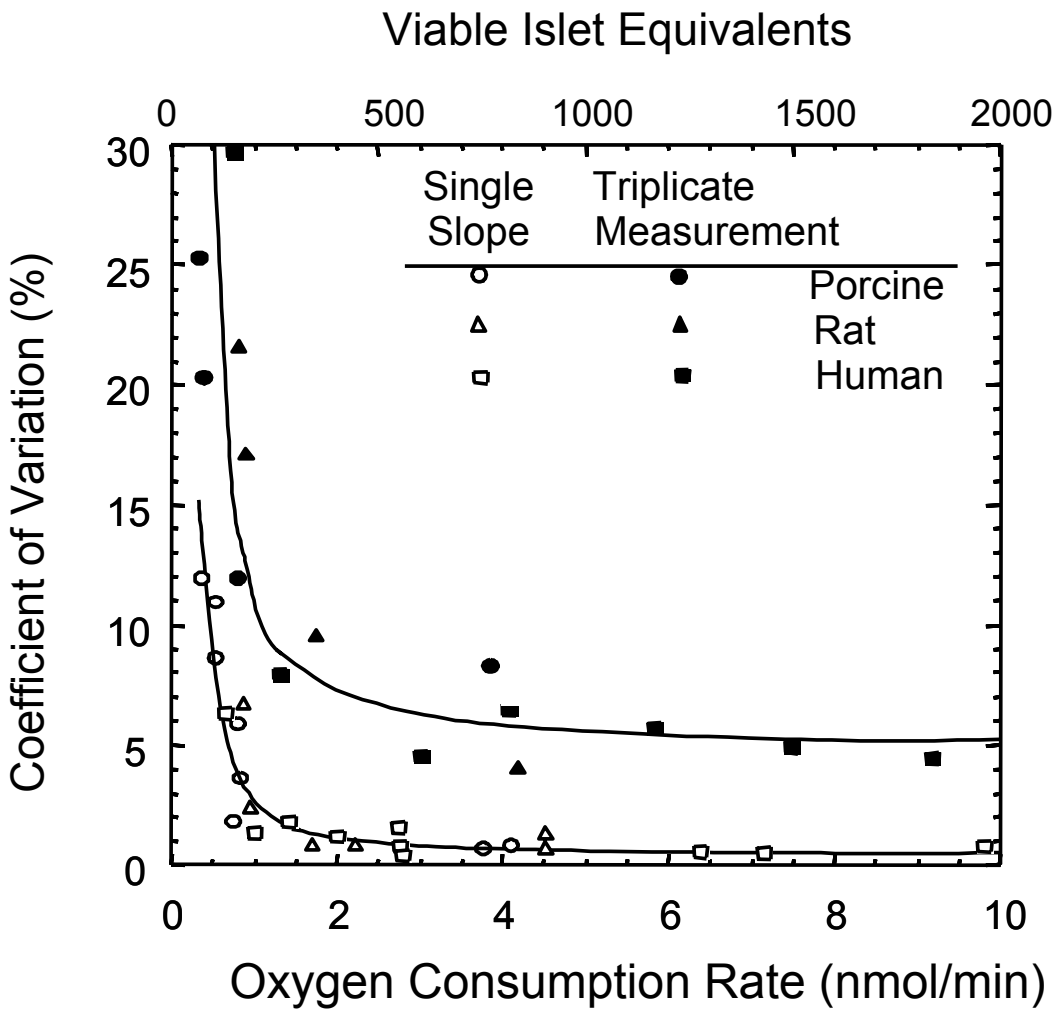
	Slope (mmHg/min)
1	13.87 ± 0.08
2	14.10 ± 0.09
3	13.51 ± 0.05

$$\frac{\text{OCR}}{\text{nucleus}} = 2.94 \pm 0.24 \frac{\text{fmol}}{\text{min nucleus}}$$

	Slope (mmHg/min)
1	4.84 ± 0.02
2	5.29 ± 0.01
3	4.93 ± 0.03

$$\frac{\text{OCR}}{\text{nucleus}} = 3.82 \pm 0.45 \frac{\text{fmol}}{\text{min nucleus}}$$

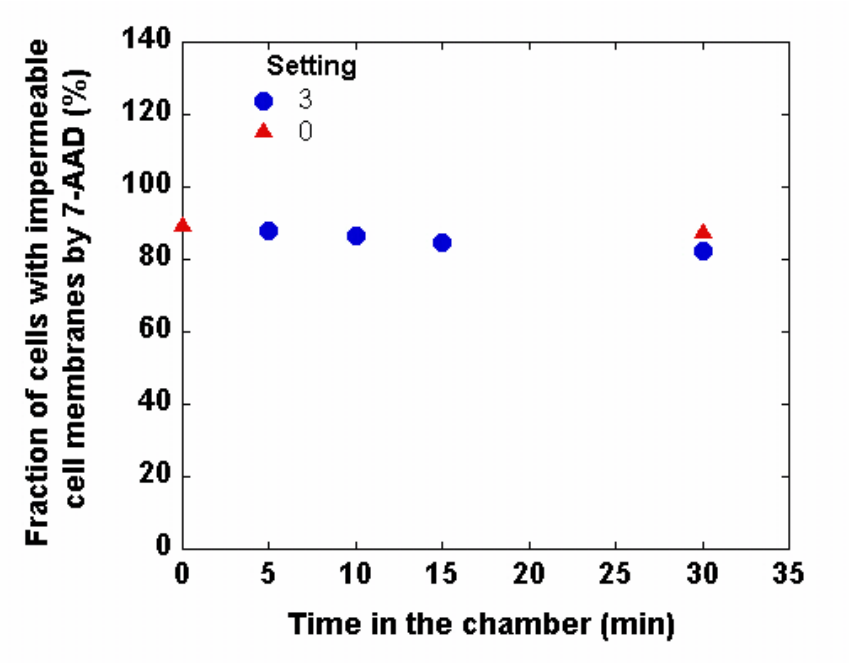
Precision of Measurements



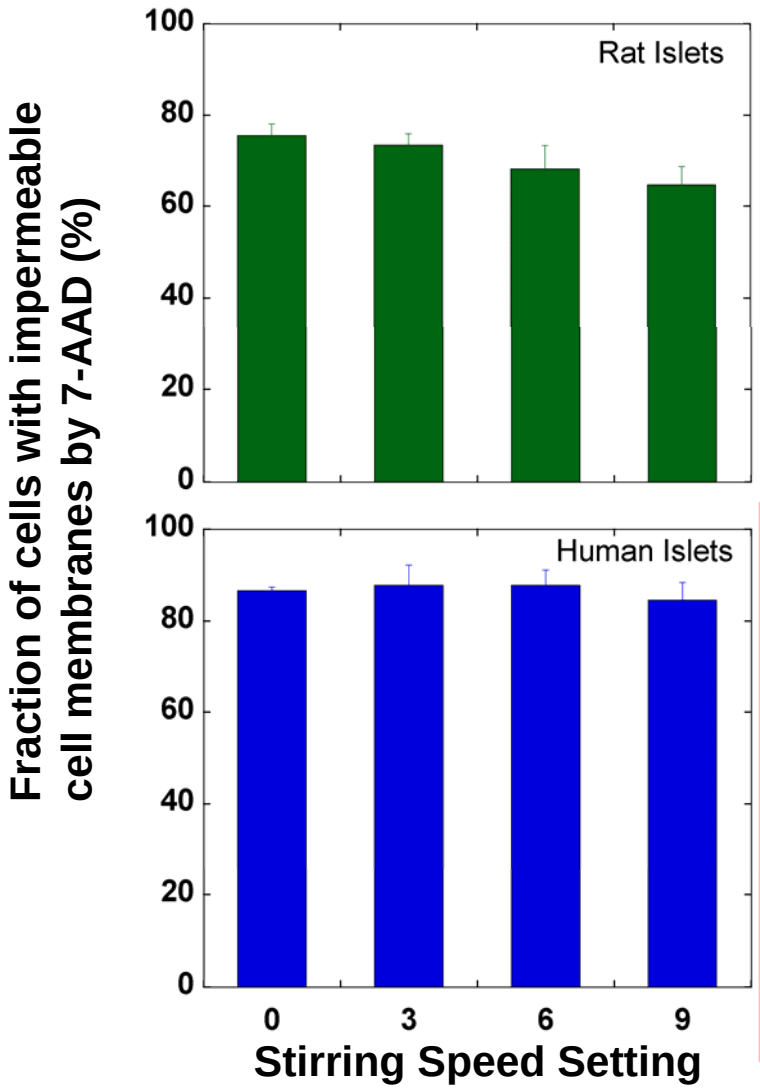
Stirring Speed Effects on Islet Membrane Integrity

Effect of Time

Rat Islets, 2 days culture 37ºC

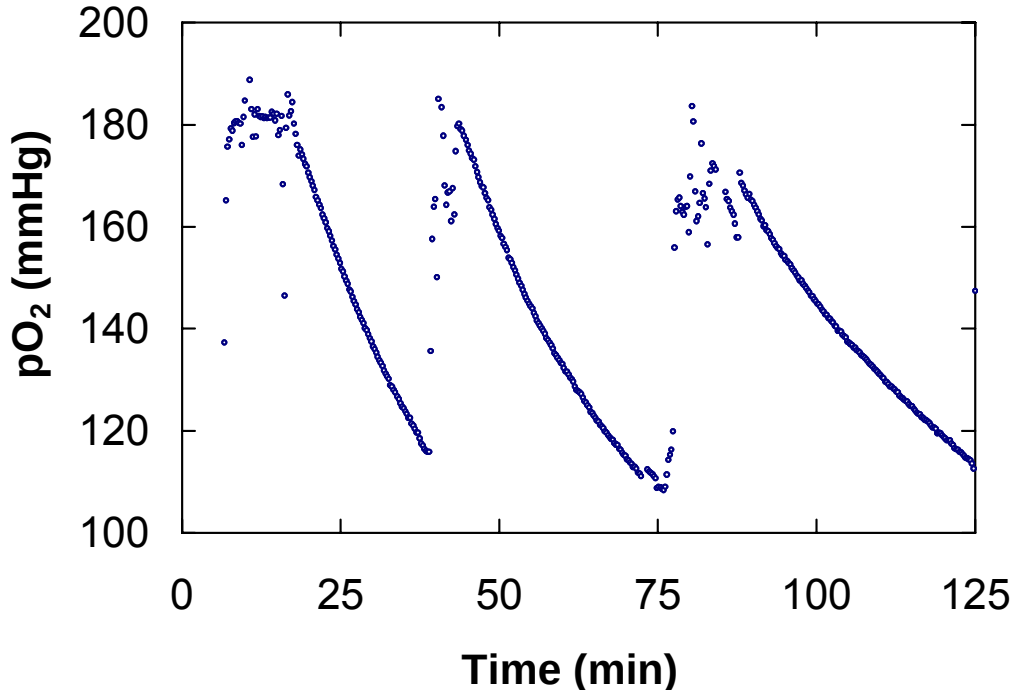


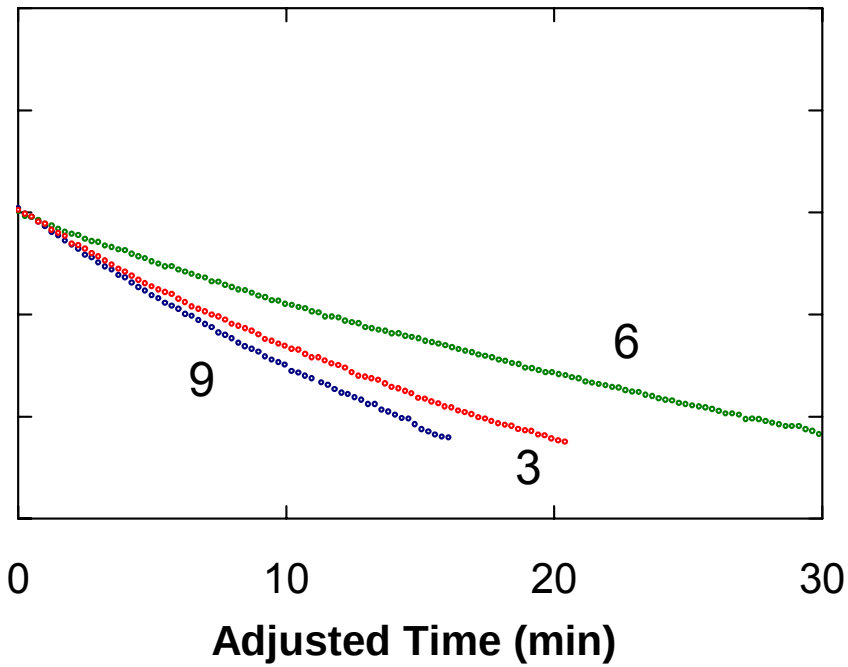
Stirring Speed Effect of Islets tested 4 hr after isolation, stirred 15 min



Curvature is Indicator of Dying Islets

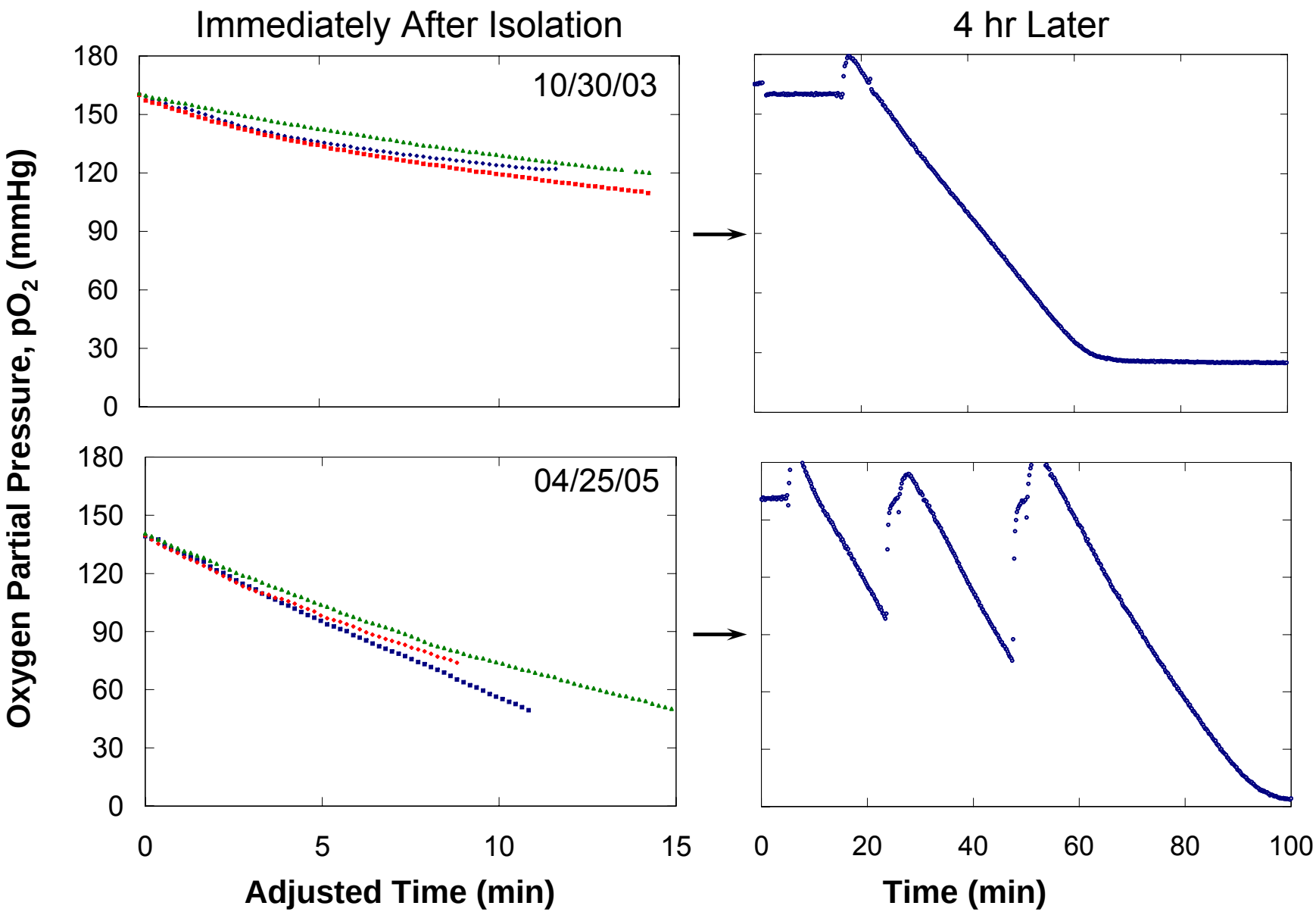
Measurements made 4 hr after isolation of rat islets





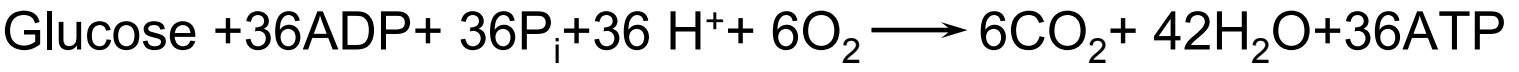
Stirrer Setting	Slope (mmHg/min) Initial	Slope (mmHg/min) Final	Fraction of cells with impermeable membranes by 7-AAD (%)
0			60.0
9	3.24	2.34	38.3
3	2.82	1.72	30.1
6	1.84	1.16	32.4

Curvature is Present Immediately After Isolation in Otherwise Viable Islets



Interpretation of Oxygen Consumption Rate Parameters

1. Oxidative Phosphorylation

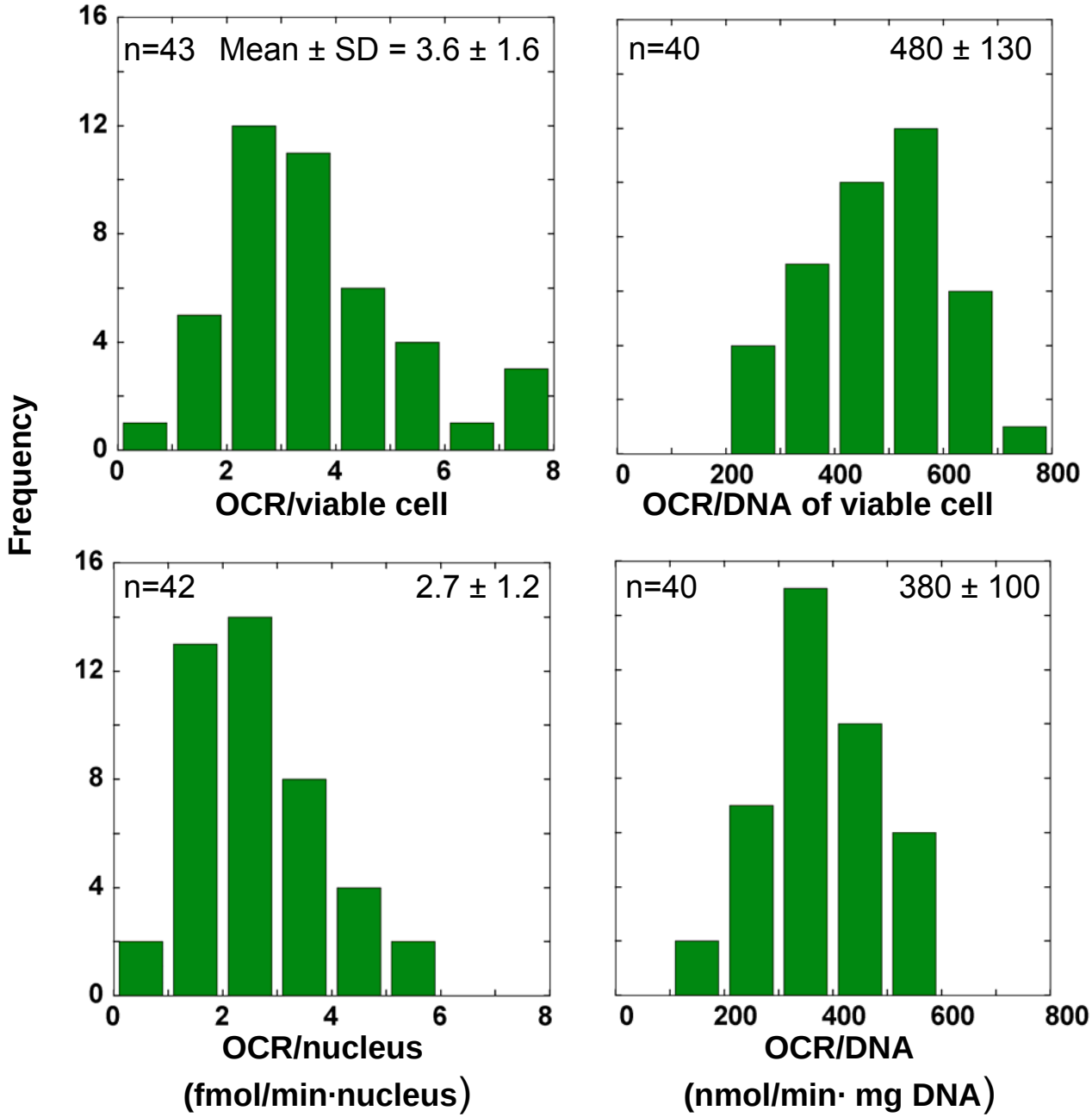


ATP Production Rate = 6 x Oxygen Consumption Rate

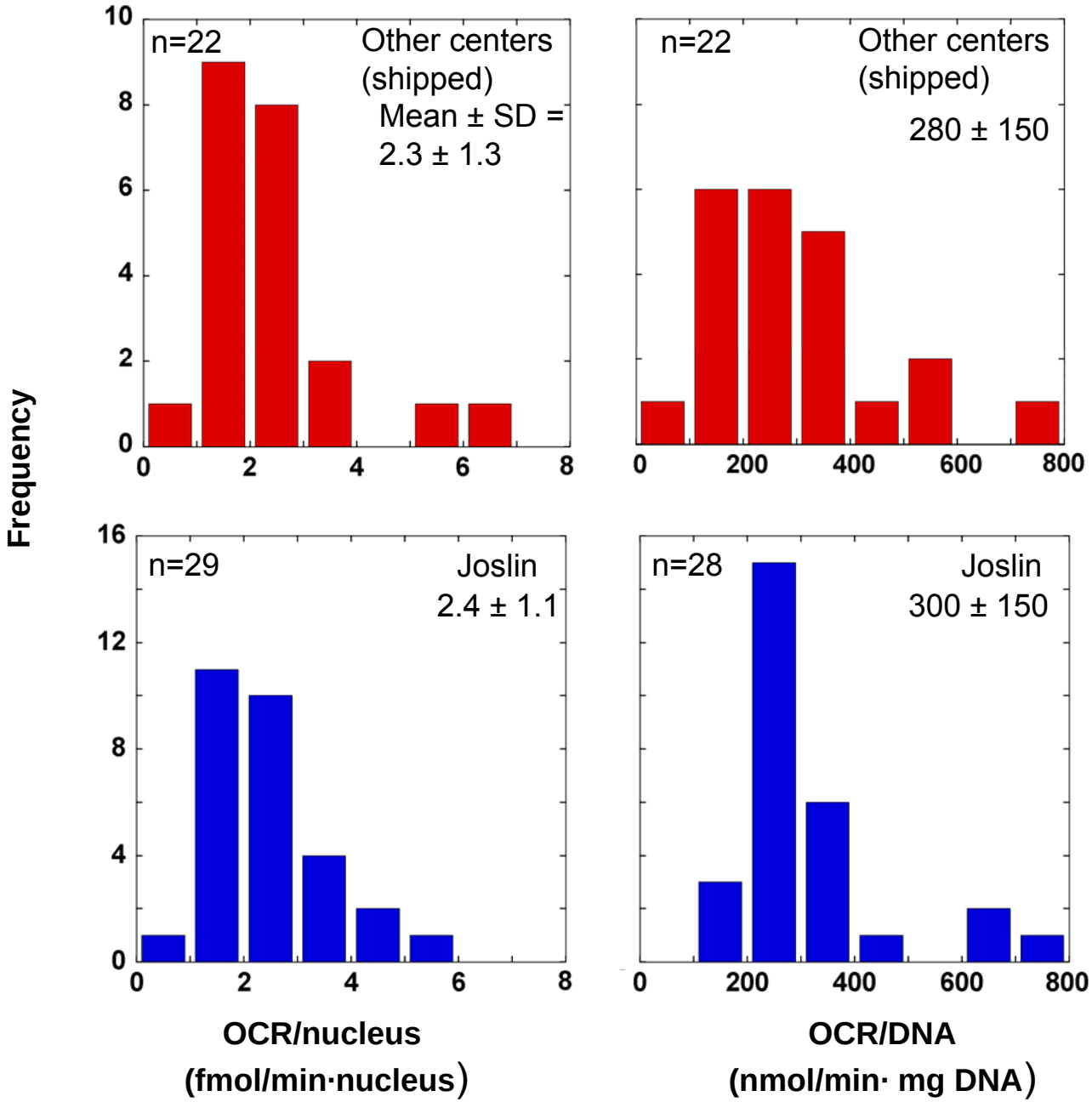
2. Assume the average OCR per viable cell under standard conditions, 37°C, DMEM, no serum is the same for all islet batches

Parameter	Proportional To	Measure of
OCR	Number of viable cells / Volume of viable tissue	Amount of good tissue
DNA	Number of cells / Total tissue volume	Total amount of tissue
$\frac{OCR}{DNA}$	$\frac{\text{Viable tissue volume}}{\text{Total tissue volume}}$	Quality of the tissue
$\frac{OCR/DNA}{(OCR/DNA)_V}$ =	Fractional Viability	Quality of the tissue

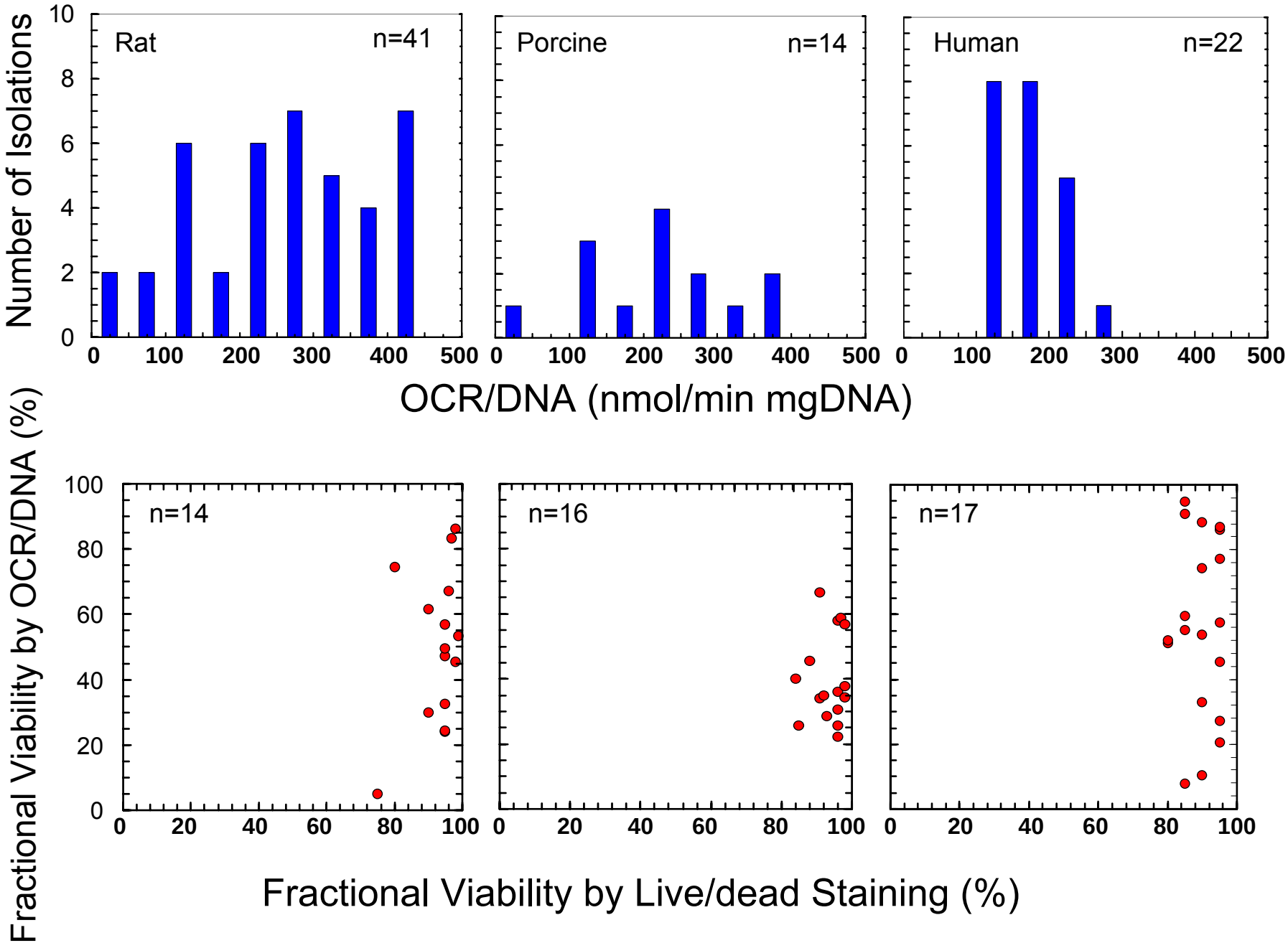
OCR/cell in Rat Islets



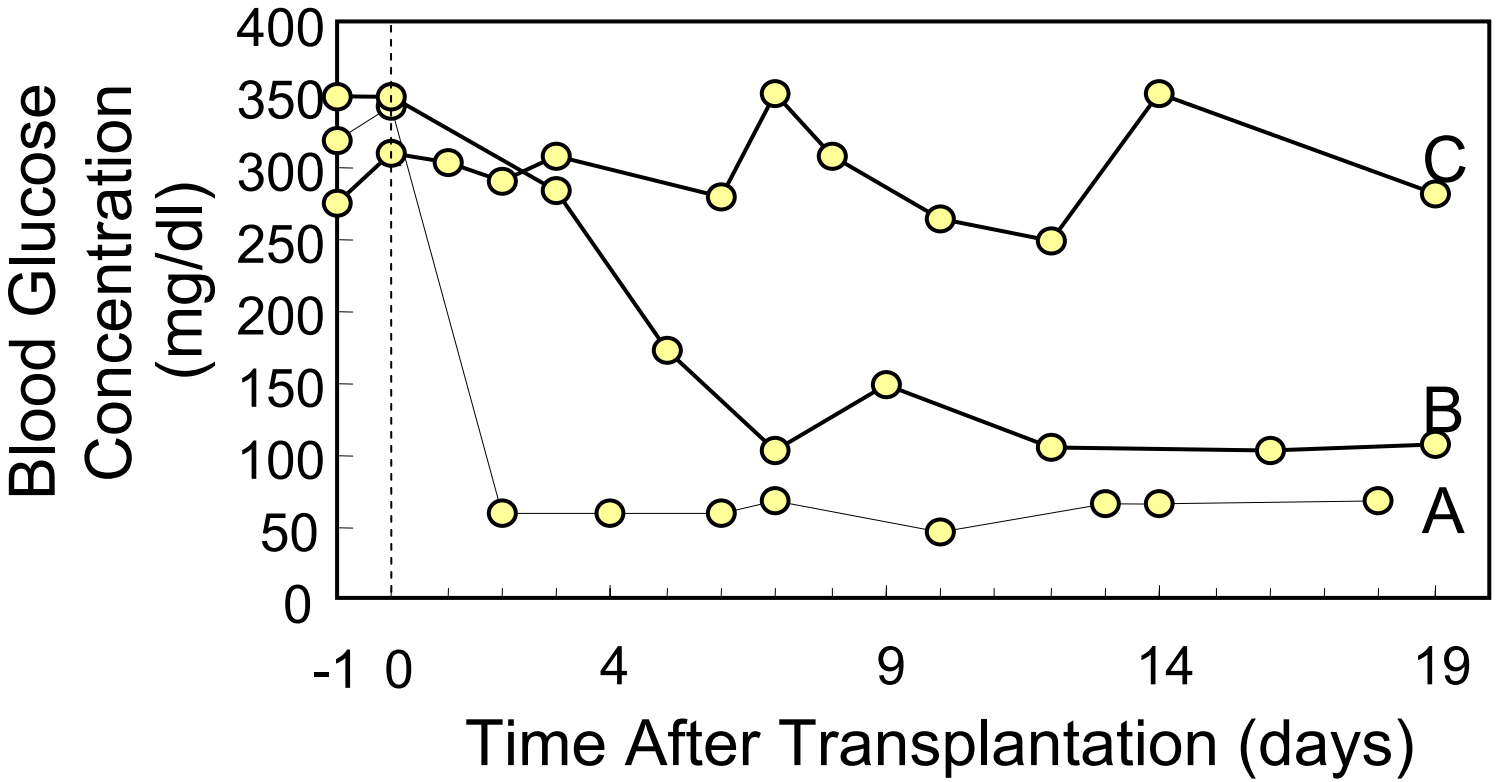
OCR/cell in Human Islets



Distribution of OCR/DNA and Comparison with FDA/PI

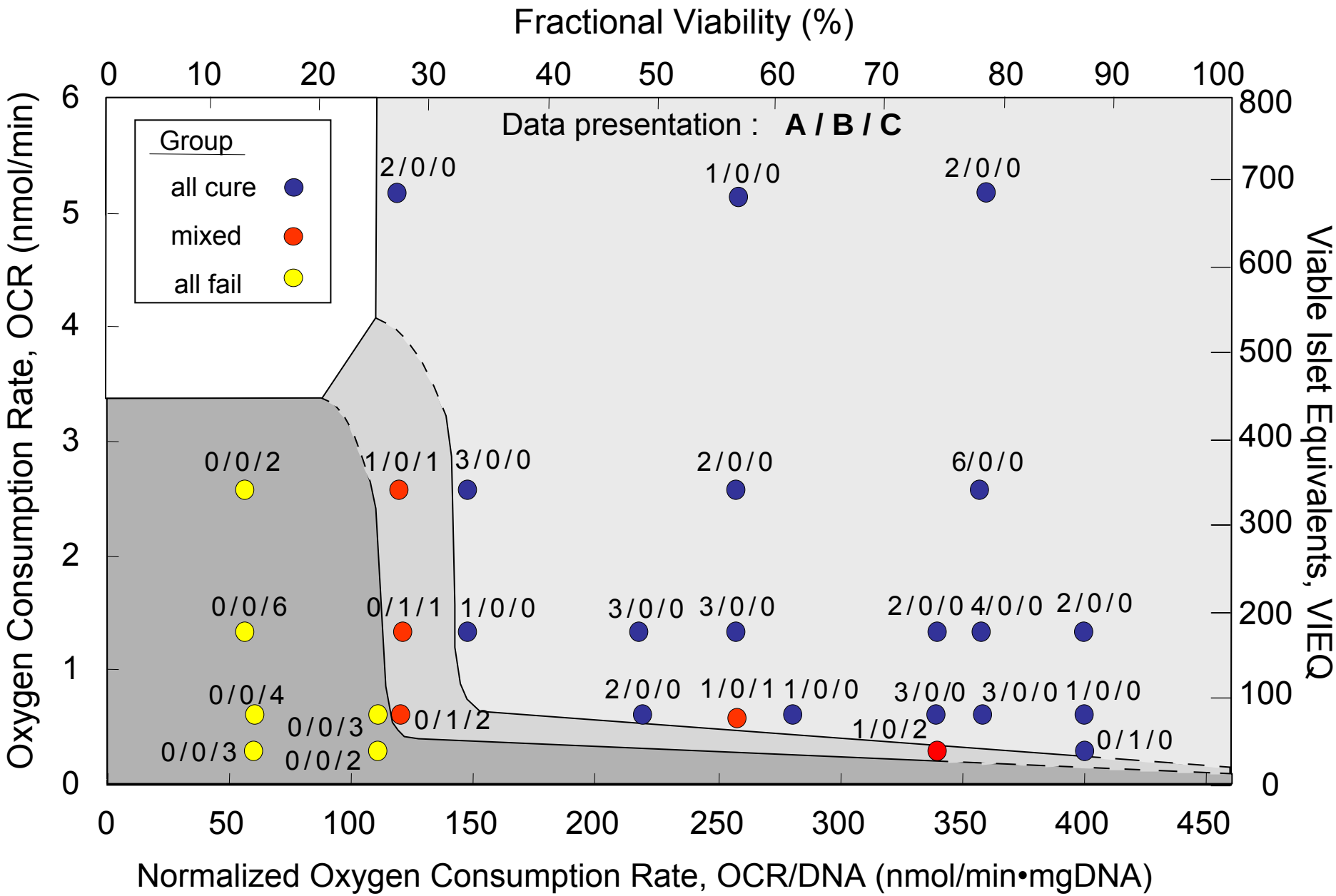


Typical Responses to Rat and Porcine Islet Transplants in Diabetic Balb/C Mice (Anti-CD4)



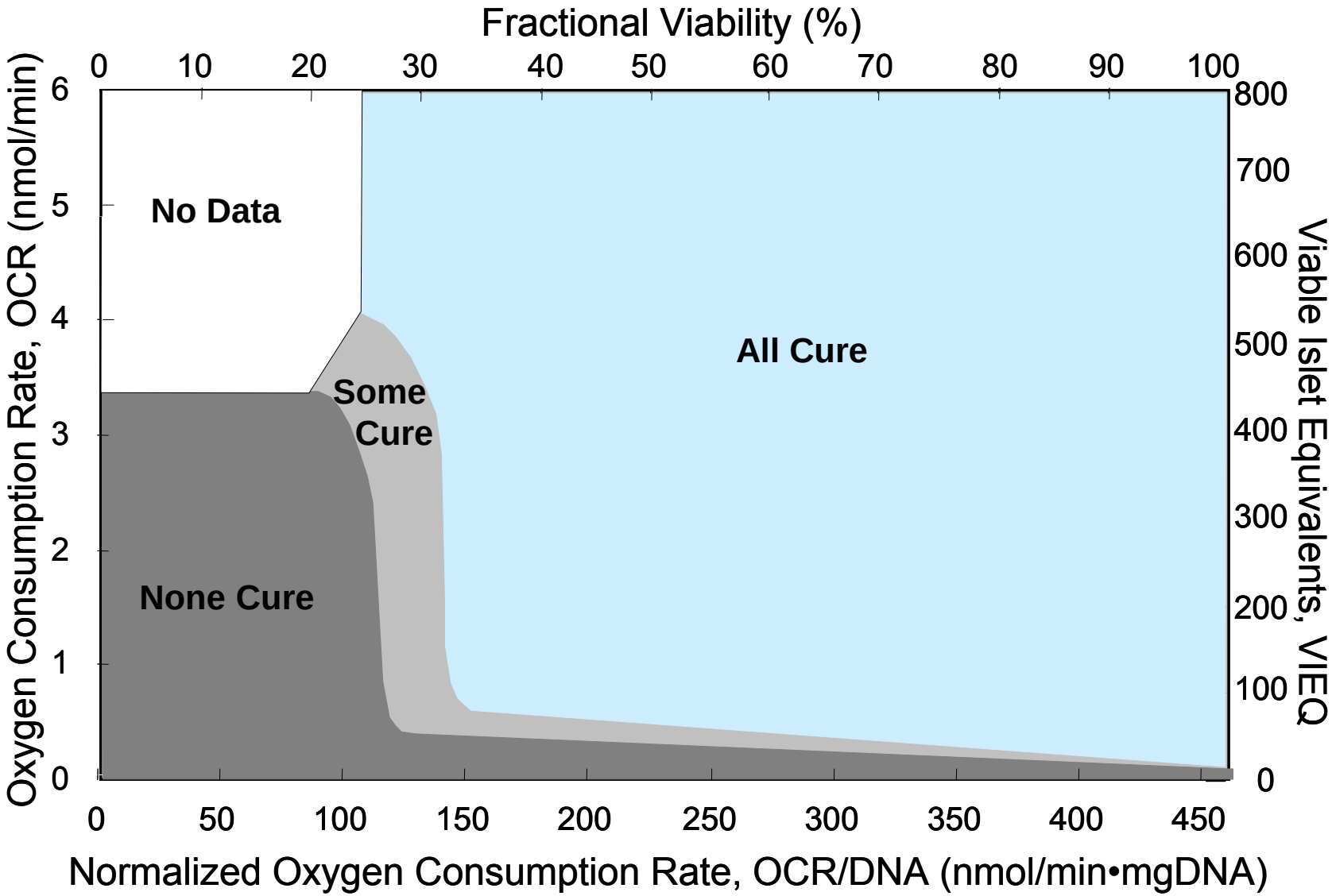
A Blood glucose ≤ 100 mg/dl for ≥ 7 days-Rapid normalization (1 - 2 days)
B Blood glucose 100 - 200 mg/dl-Some with delayed normalization
C Blood glucose > 200 mg/dl (usually > 300 mg/dl)

Response to Rat Islet Transplants in Diabetic Balb/C Mice (Anti-CD4)



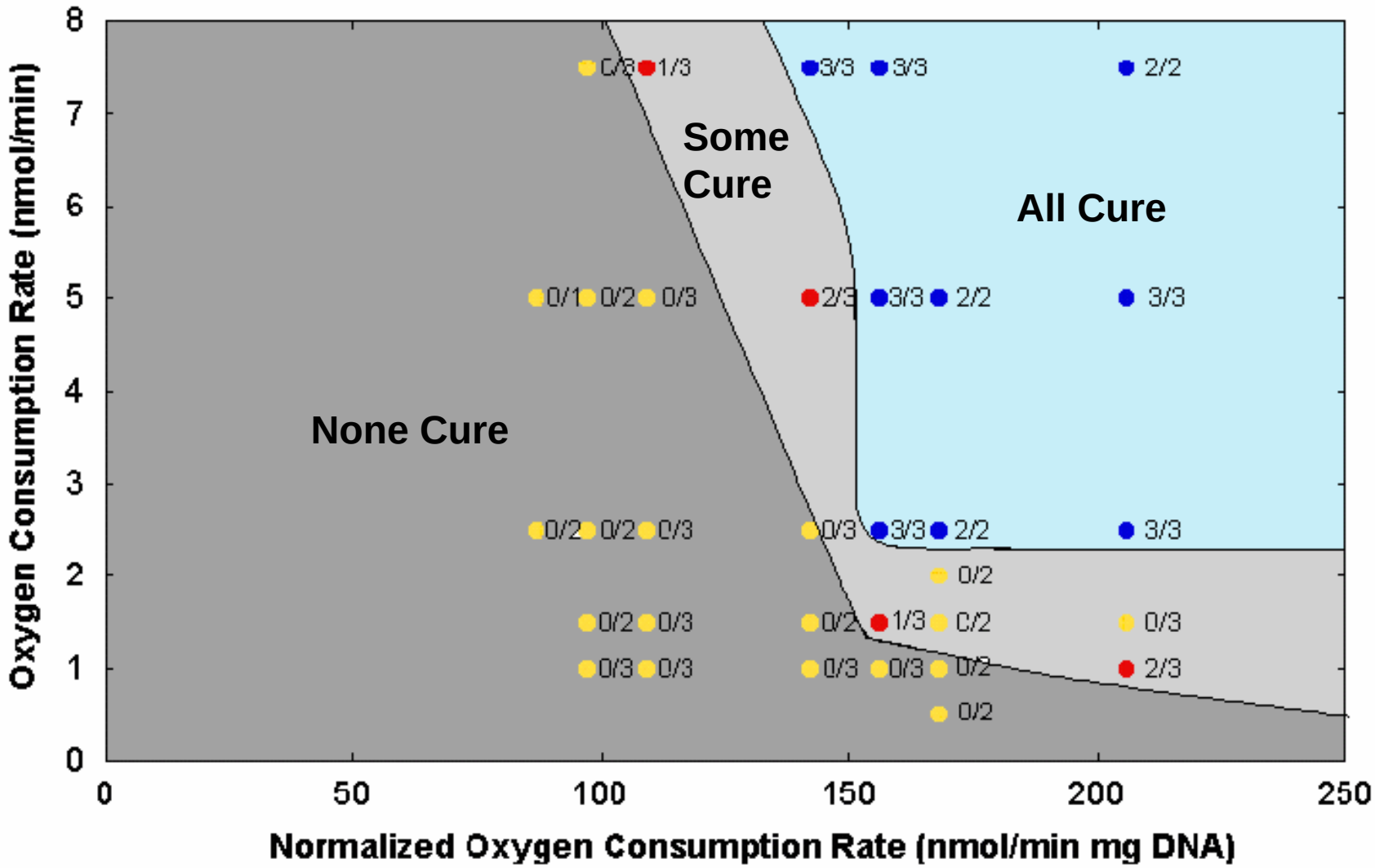
OCR Measurements Can Predict Transplantation Outcome

Rat islets transplanted into kidney capsule of immunosuppressed diabetic BalbC mice



Response to Human Islet Transplants in Diabetic Immunodeficient Mice

Human islets were taken from the highest purity fraction (>90% by DTZ)



Stimulation of OCR by Exogenous Substrates

Stimulated OCR: PBS 37°C after the addition of glucose
Basal OCR: PBS 37°C, no glucose

Tissue	Species	n	Stimulated OCR / Basal OCR Glucose 20 mM
Islets	Rat	9	1.58 ± 0.14
	Human	6	1.48 ± 0.13
	Porcine	3	1.49 ± 0.30
Exocrine	Rat	1	1.0
	Human	3	0.90 ± 0.10
	Porcine	2	1.0

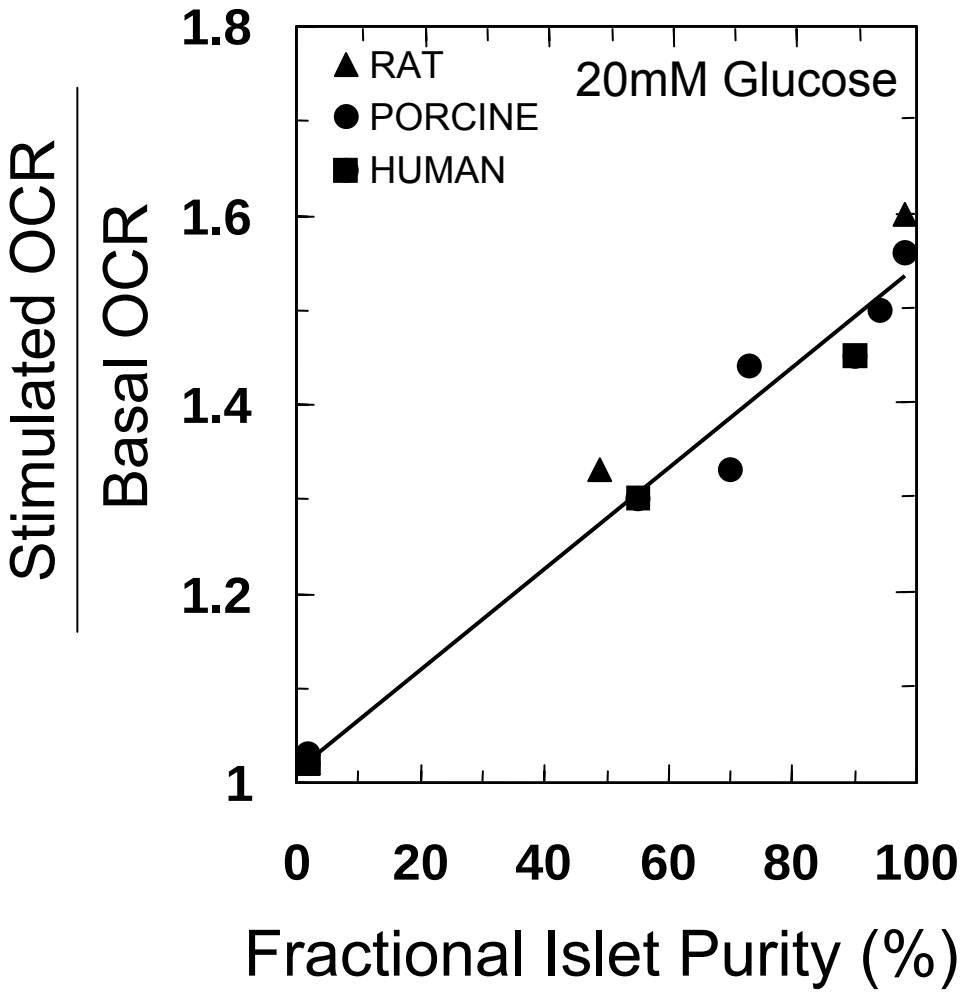
Similar measurements in DMEM, no serum

Islets	Rat	5	1.16*

* Entire increase occurred between 0 and 3 mM glucose

Stimulation Ratio in Prepared Islet and Exocrine Mixtures

Basal conditions: PBS, 37ºC, no exogenous substrates

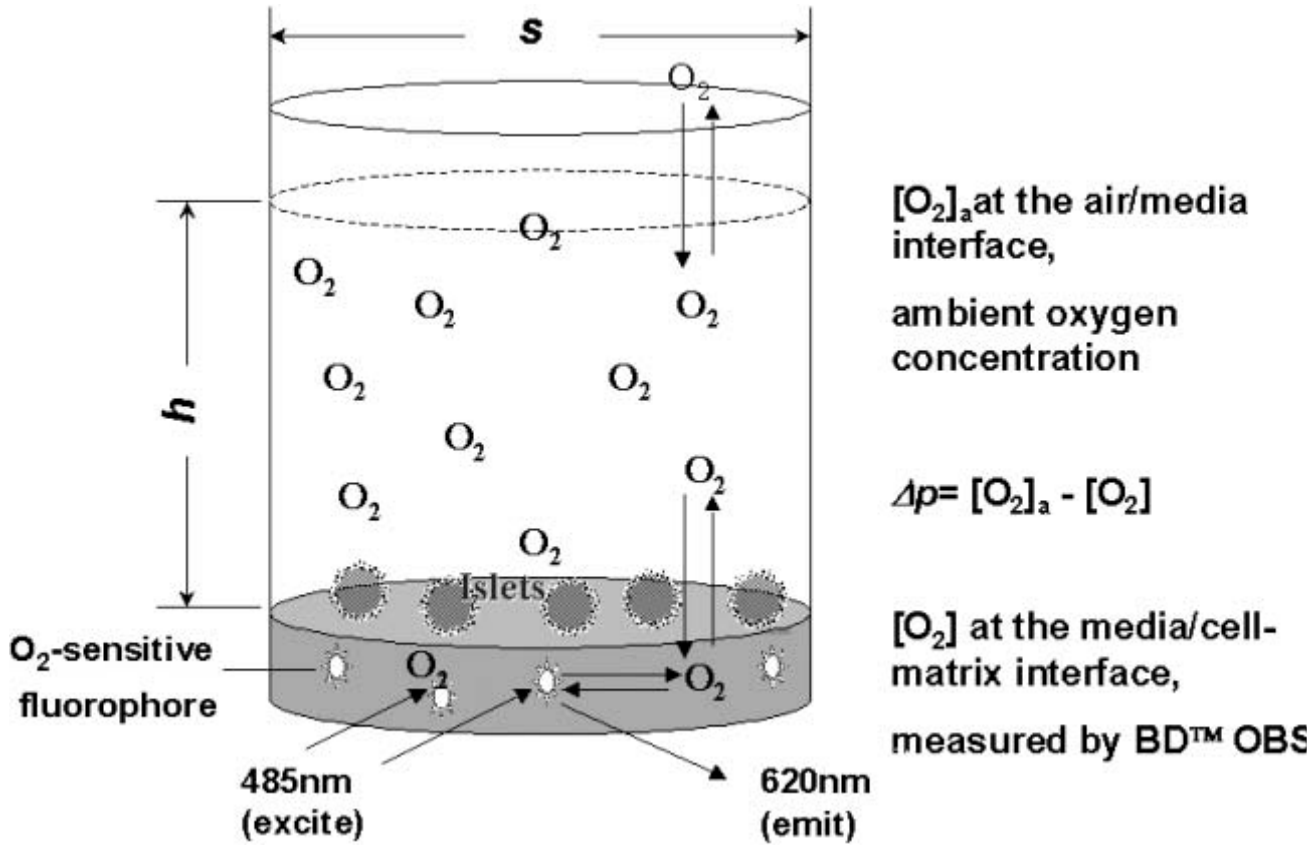


OCR Measurements with Instech Stirred Chamber

Conclusions

1. The Instech stirred tank system provides rapid, accurate, and precise measurement of the OCR of islet preparations.
2. It has been used reliably in our laboratory by about 10 technical staff for over 500 measurements with about 100 islet preparations.
3. OCR measurements obtained with the Instech system are predictive of transplantation outcome in immuno-deficient diabetic mice transplanted with rat islets and high purity (>90% DTZ) human islet preparations.

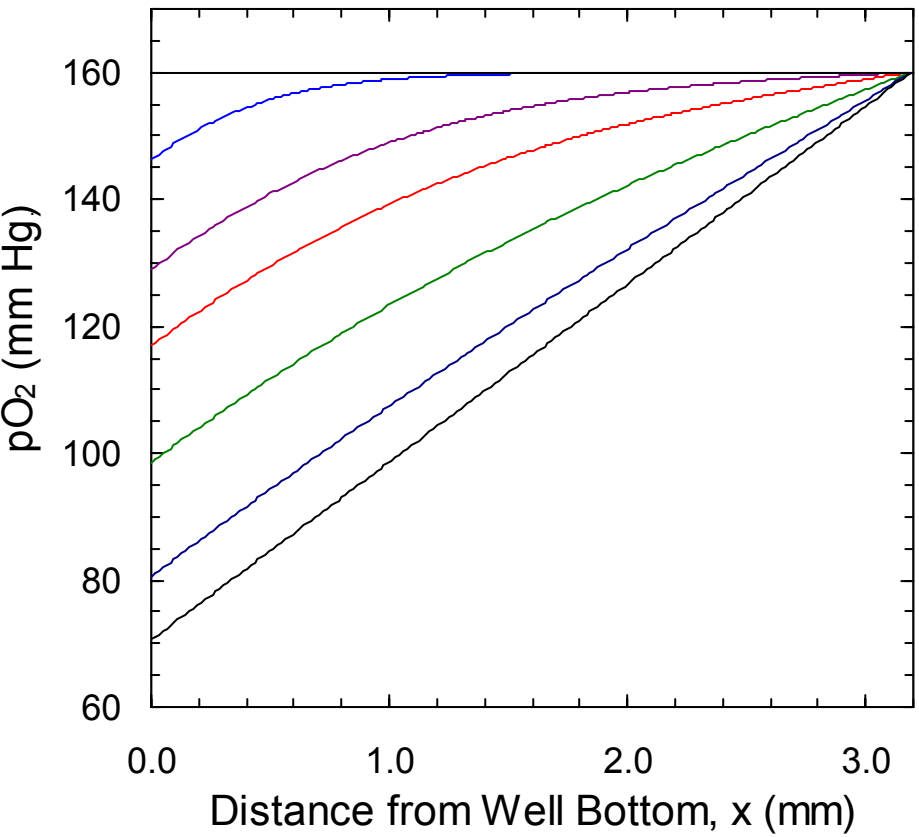
Schematic Representation of BD OBS Well Containing Islets



From: Wang W, Upshaw L, Strong DM, Robertson RP, and Reems J., "Increased oxygen consumption rates in response to high glucose detected by a novel oxygen biosensor system in non-human primate and human islets," *J. Endocrinology*, **185**, 445-455 (2005).

Development of Oxygen Profiles in BD OBS

250,000 Jurkat cells in 100 μl of culture medium within an idealized OBS well
(OCR/cell = 0.84 fmol/min cell)



Calculation of OCR at steady state

$$OCR = \frac{D \cdot \alpha \cdot A}{L} \cdot (\Delta pO_2)$$

D = Diffusivity of oxygen in water

α = Bunsen solubility coefficient of oxygen in water

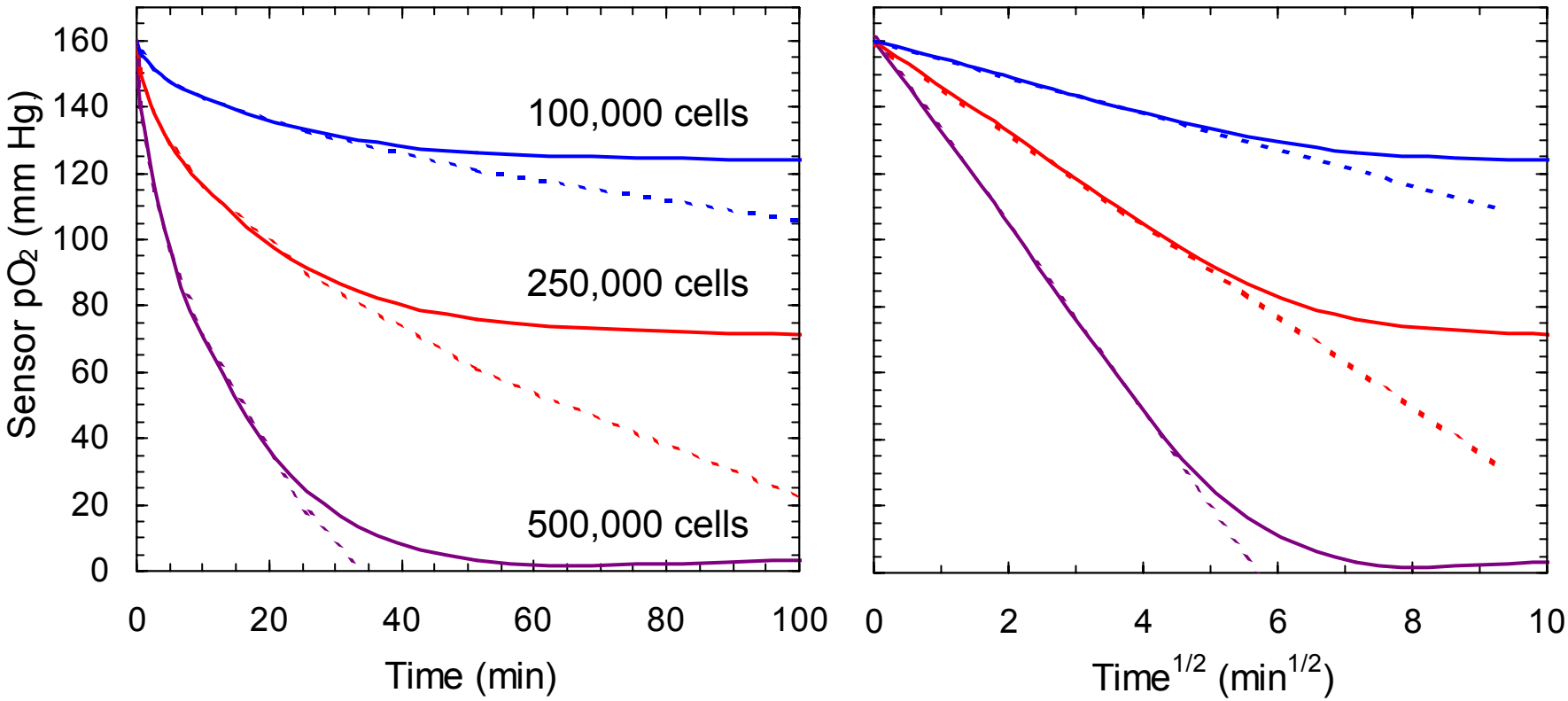
A = Area of well

L = Height of liquid

$\Delta pO_2 = pO_2$ (ambient) - pO_2(surface, x=0)

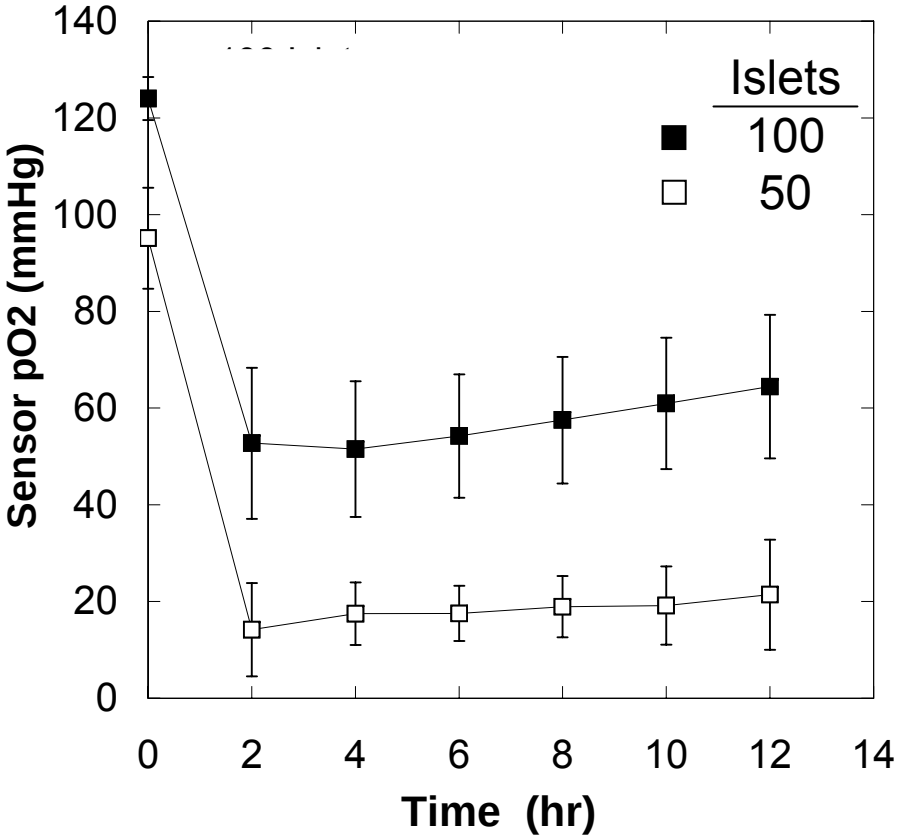
Theoretical Prediction of Sensor Oxygen Partial Pressure in Idealized Well

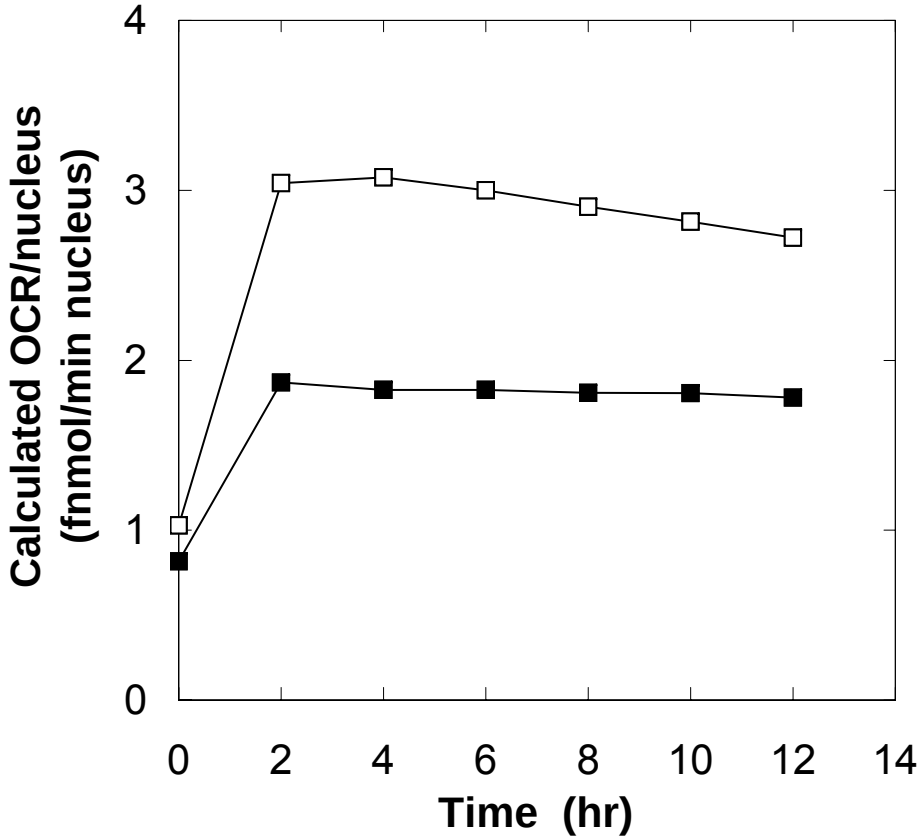
Jurkat cells in 100 μl of culture medium (OCR/cell = 0.84 fmol/min cell)



——— Full theory - - - - Theory for short time

OCR Measurement with Human Islets in BD OBS





Comparison of Initial OCR Values Obtained with the Stirred Tank and BD OBS*

	OCR (fmol/min cell)		
Cell type	Stirred tank	OBS	Ratio
Jurkat (human lymphocyte)	0.84	0.38	2.2
INS-1 (rat insulinoma)	3.0	1.3	2.3
Human islets	5.3		
50 per test		3.0	1.8
100 per test		1.9	2.9

*Approximately followed procedure of Guarino et al., 2004

Why are the Results Different?

Stirred vessel directly measures of oxygen ***consumption***

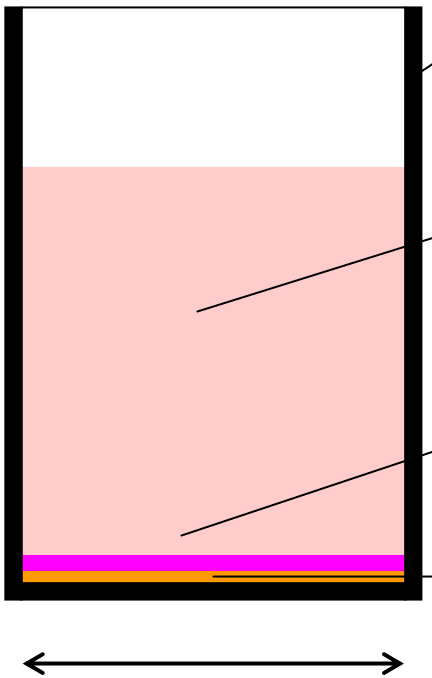
OBS plate directly measures ***average pO_2*** at a surface

- Determination of oxygen consumption rate requires use of the integrated form of Fick's law of diffusion applicable at steady state
- Application of Fick's law invokes many assumptions
- Results are only as good as the assumptions that are made

Assumptions Required for OCR Determination with OBS Plate at Steady State

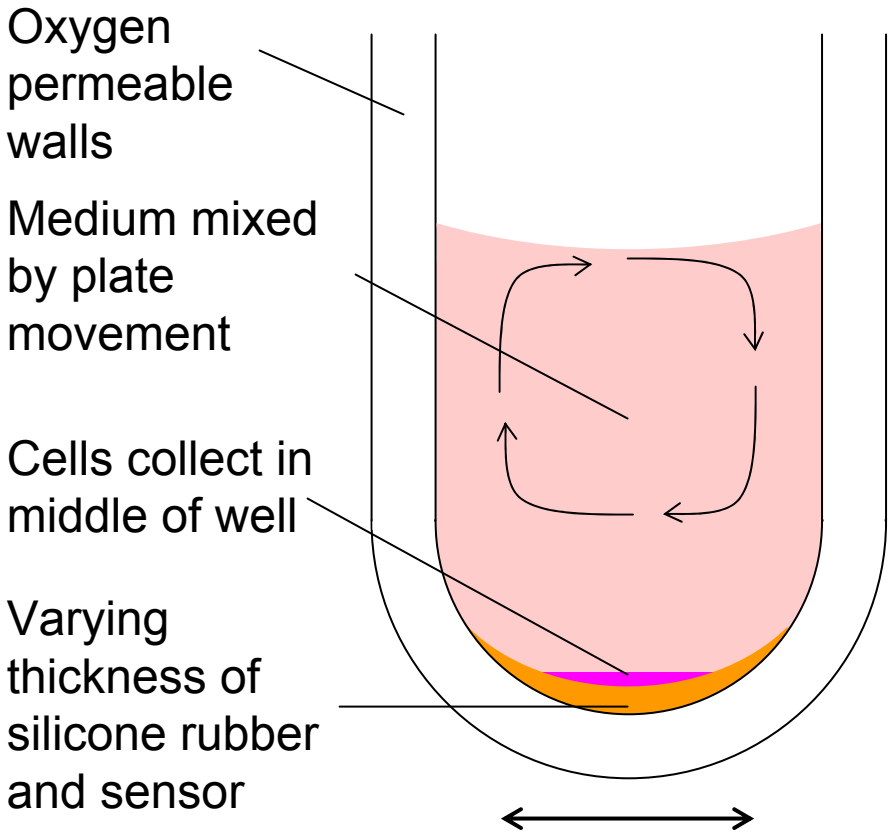
Idealized Well (assumed)

Perfect cylinder



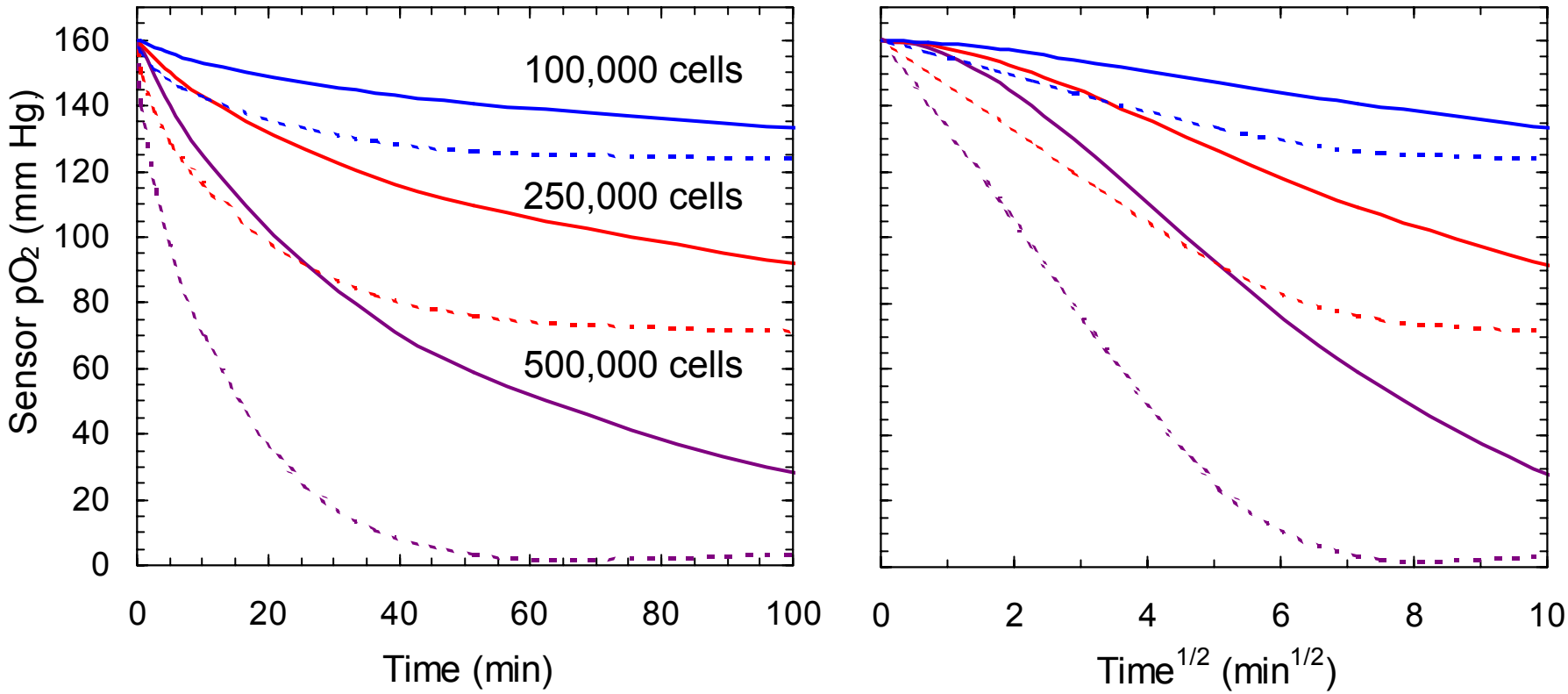
Actual Well

Round bottomed well



Theoretical Prediction of Sensor Oxygen Partial Pressure in Actual Well

Jurkat cells in 100 μl of culture medium (OCR/cell = 0.84 fmol/min cell)

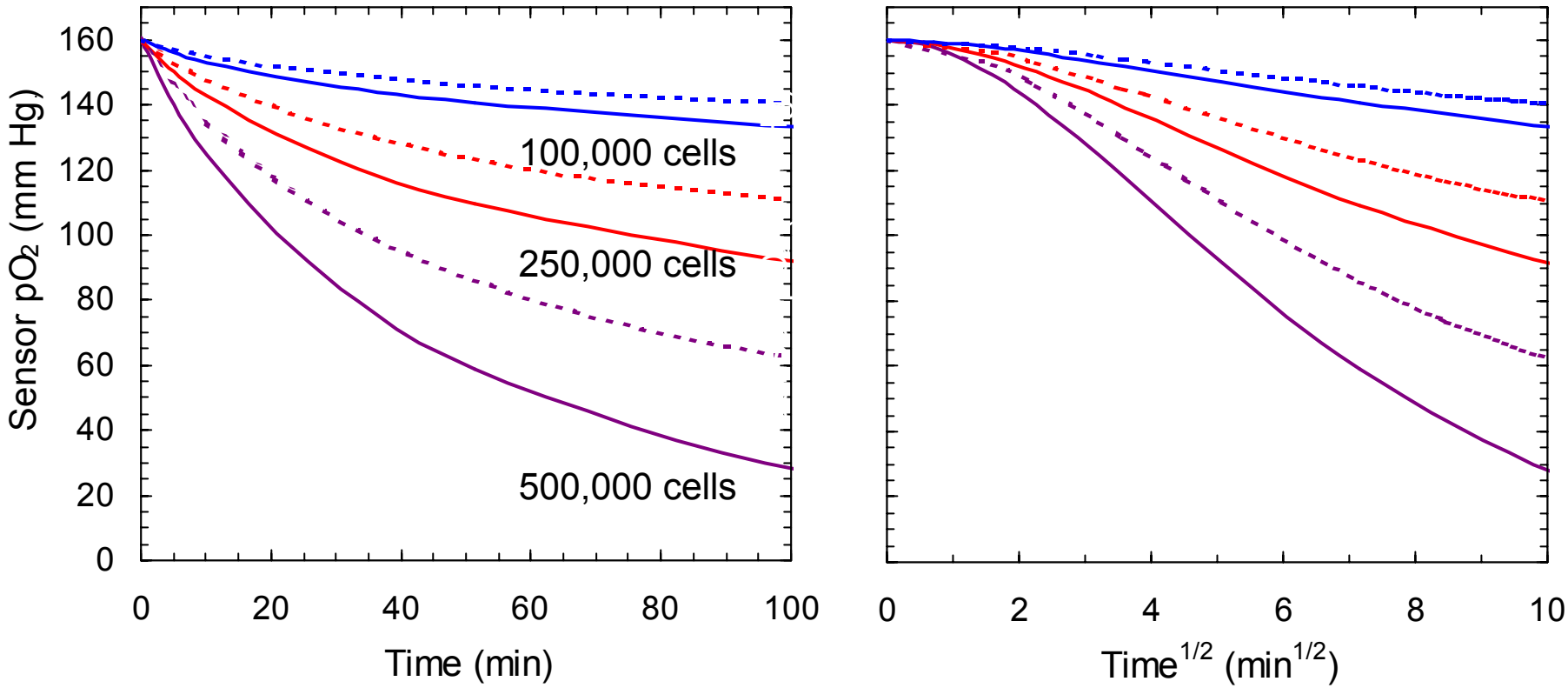


Real Well

Idealized Well

Theoretical Prediction of Sensor Oxygen Partial Pressure in Actual Well

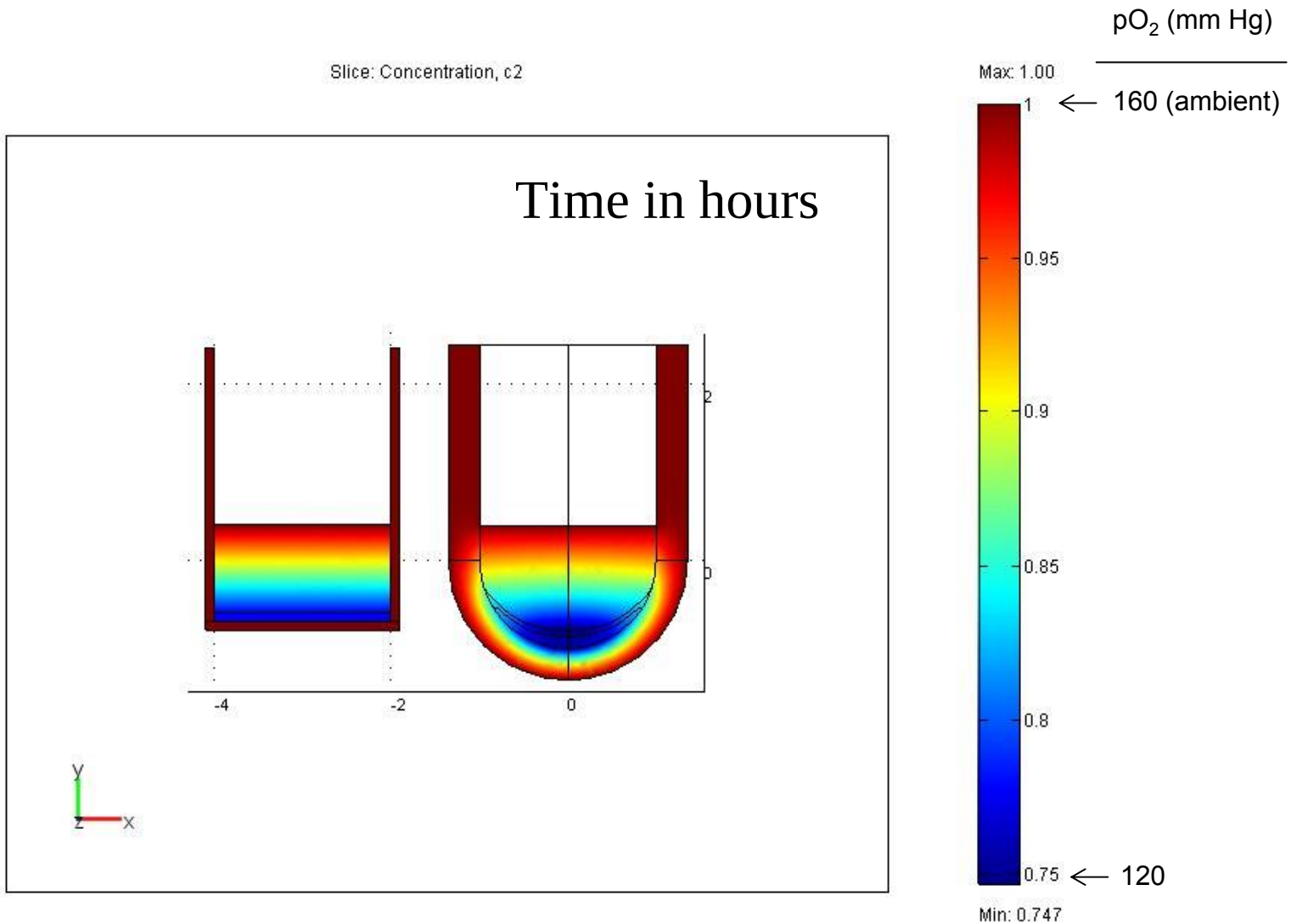
Jurkat cells in 100 μl of culture medium (OCR/cell = 0.84 fmol/min cell)



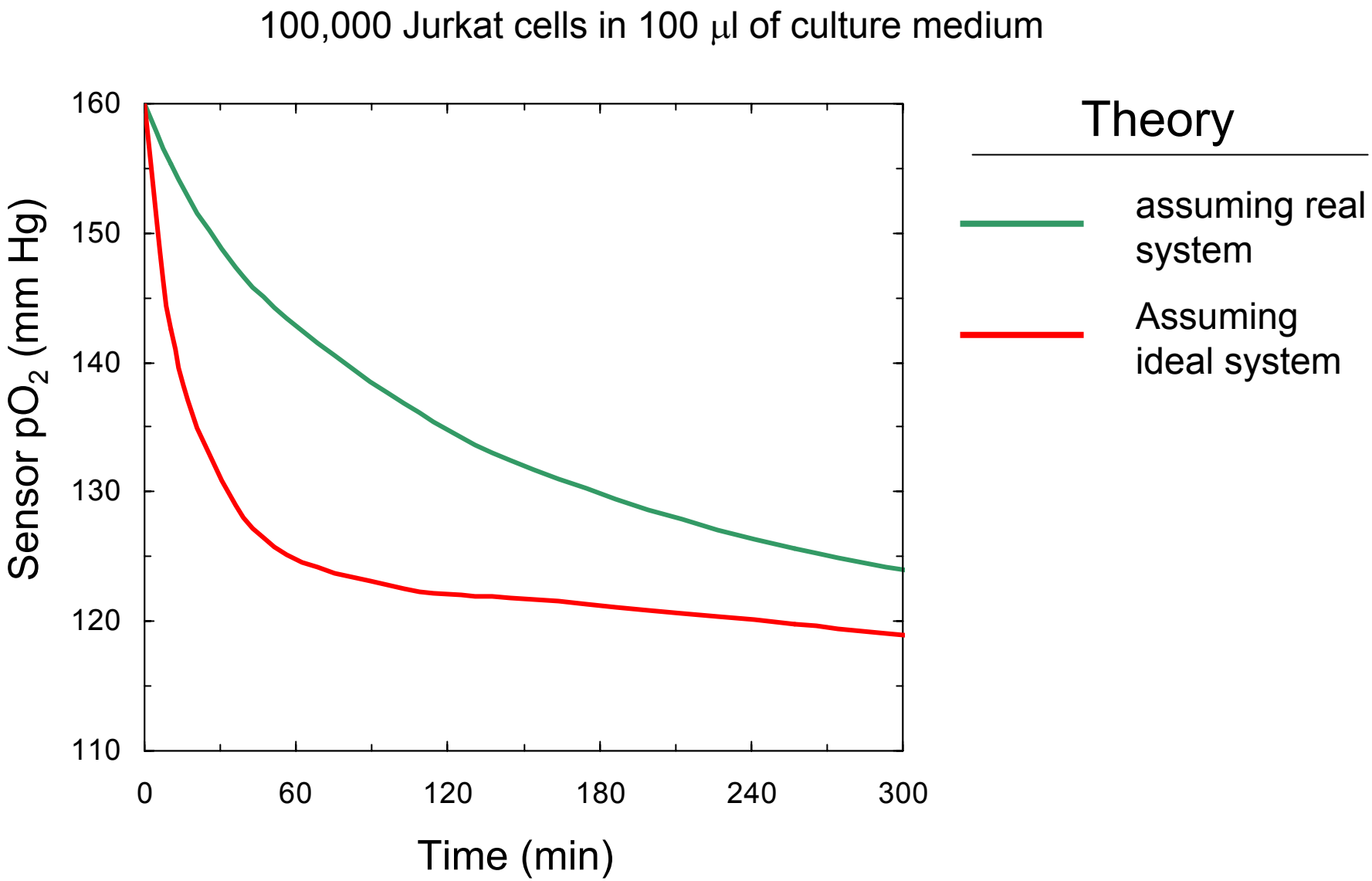
——Rounded Well - - - - Flat Well

Computer Simulation of Ideal and Real Wells

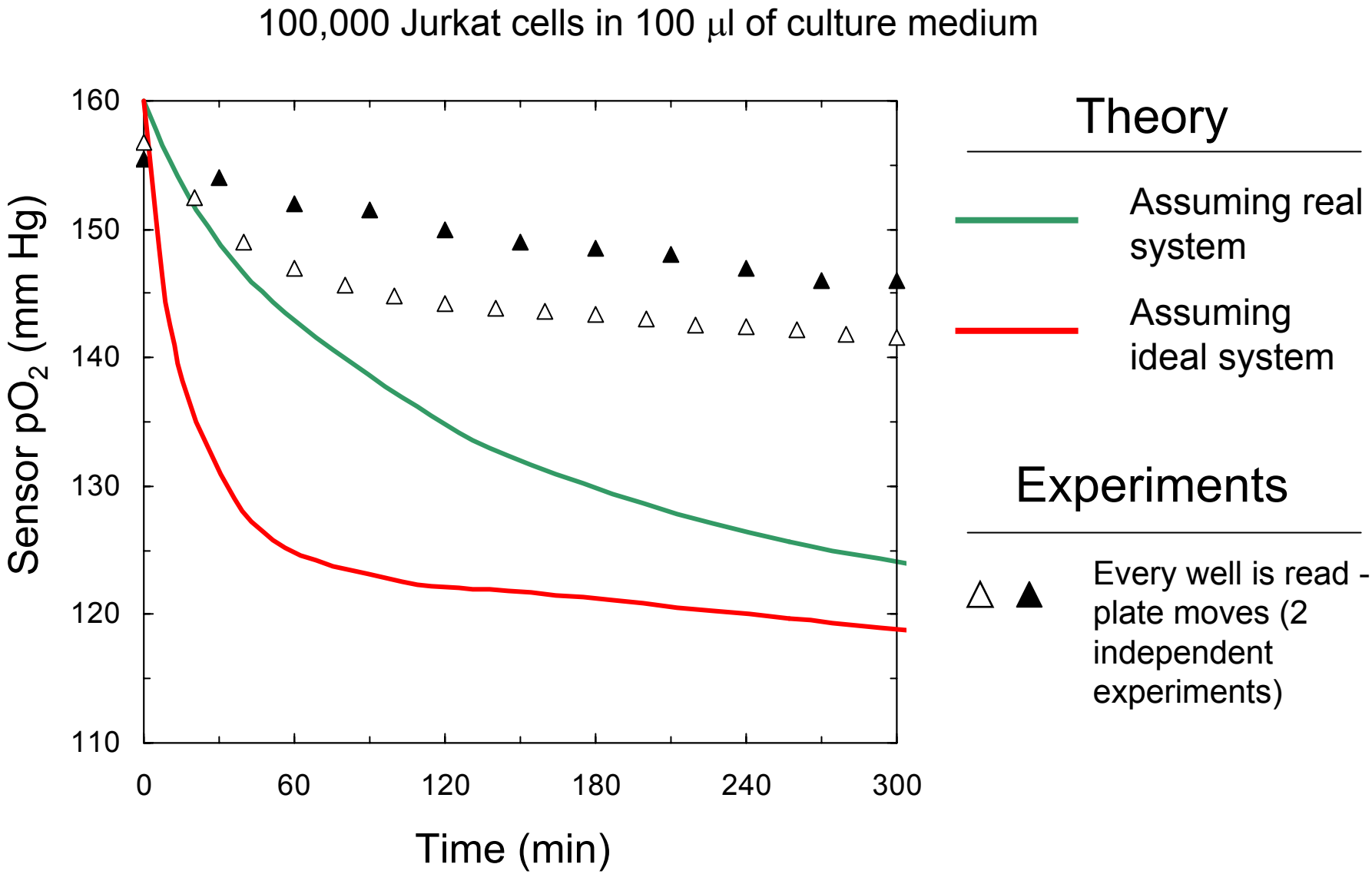
100,000 Jurkat cells (doubling time = 1 day) in oxygenated medium placed in each well at time = 0



Transient Response in OBS Well

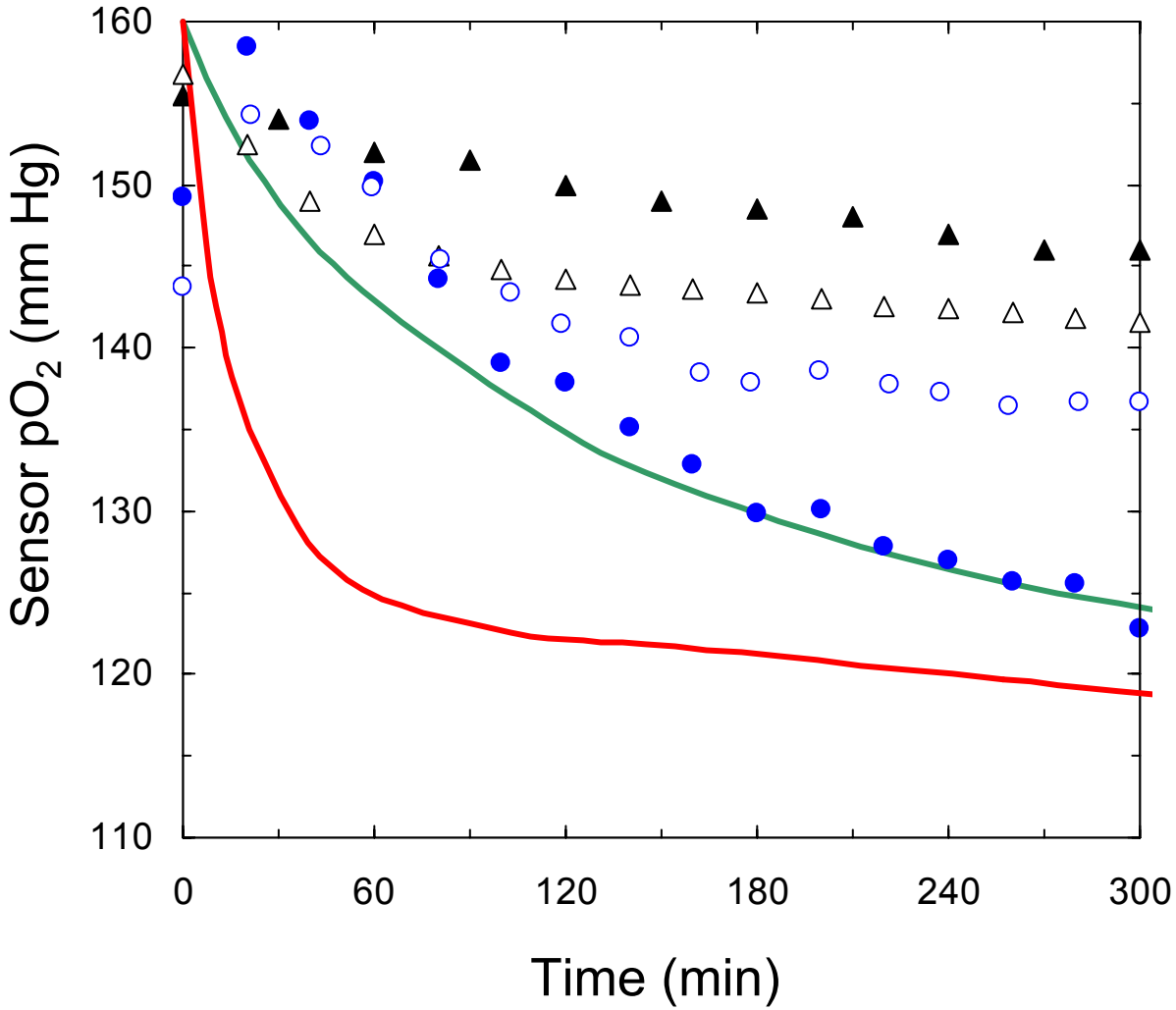


Transient Response in OBS Well



Transient Response in OBS Well

100,000 Jurkat cells in 100 μl of culture medium



Theory

Assuming real system

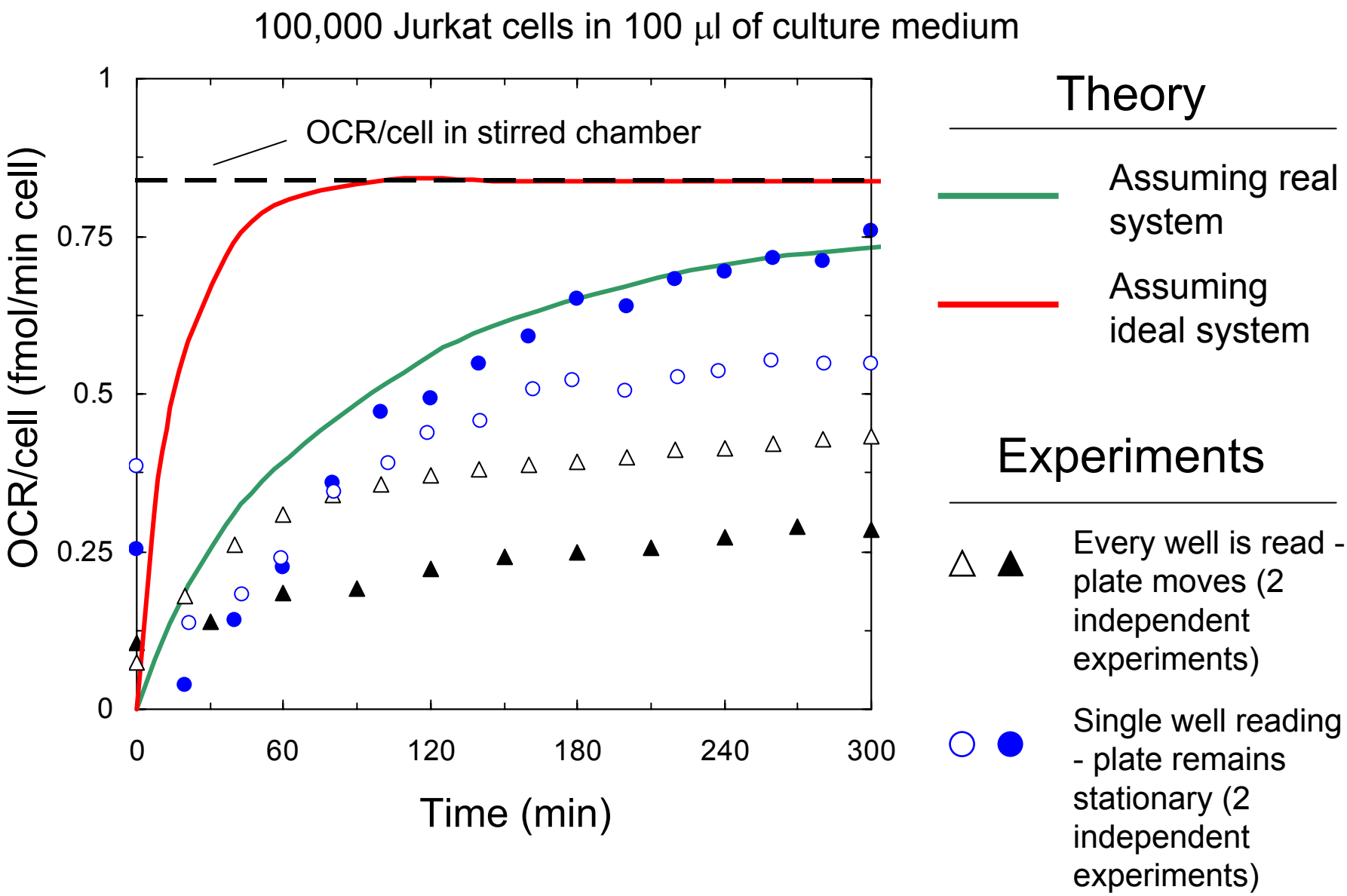
Assuming ideal system

Experiments

△ ▲ Every well is read - plate moves (2 independent experiments)

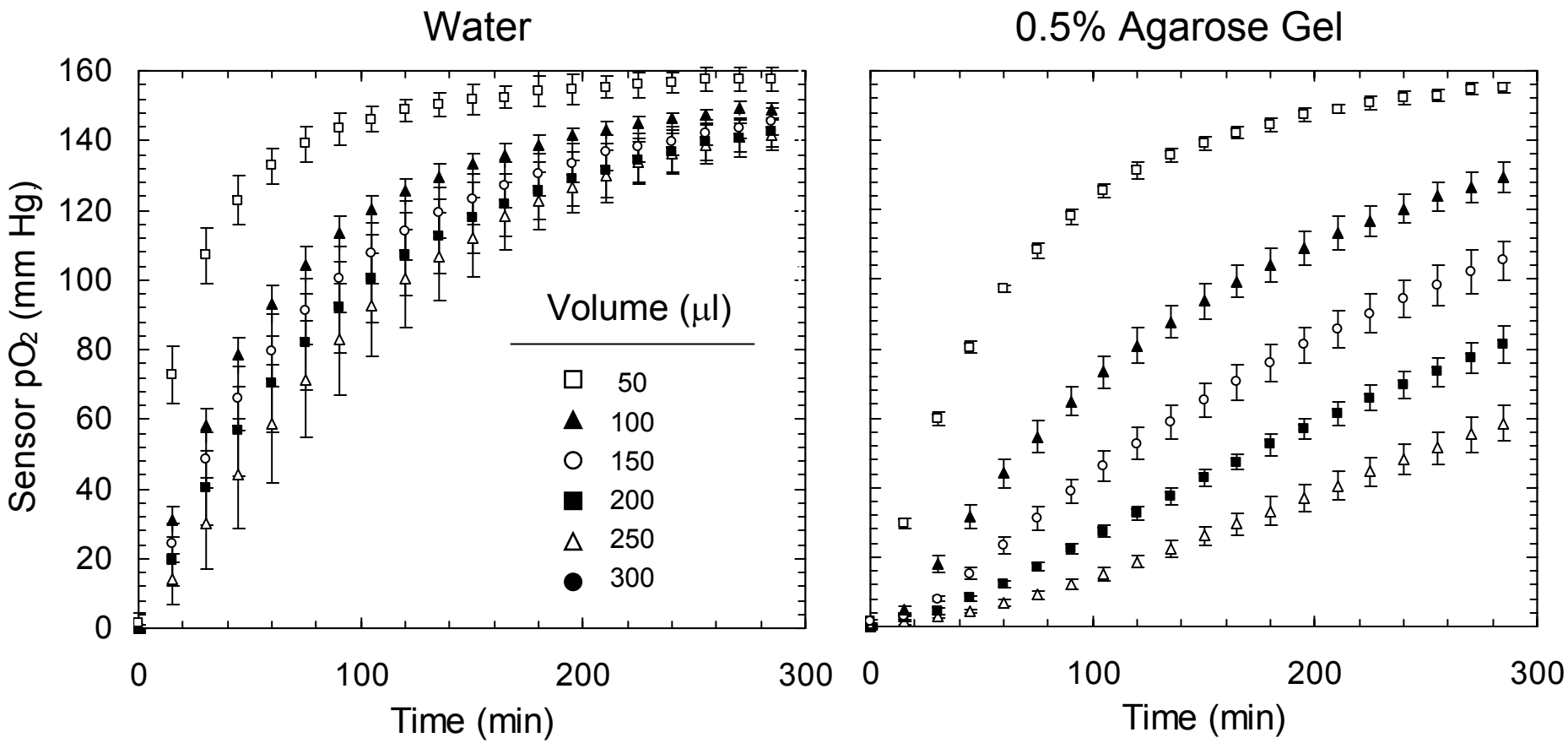
○ ● Single well reading - plate remains stationary (2 independent experiments)

Computed OCR/cell from pO_2 Values (using Fick's Law)



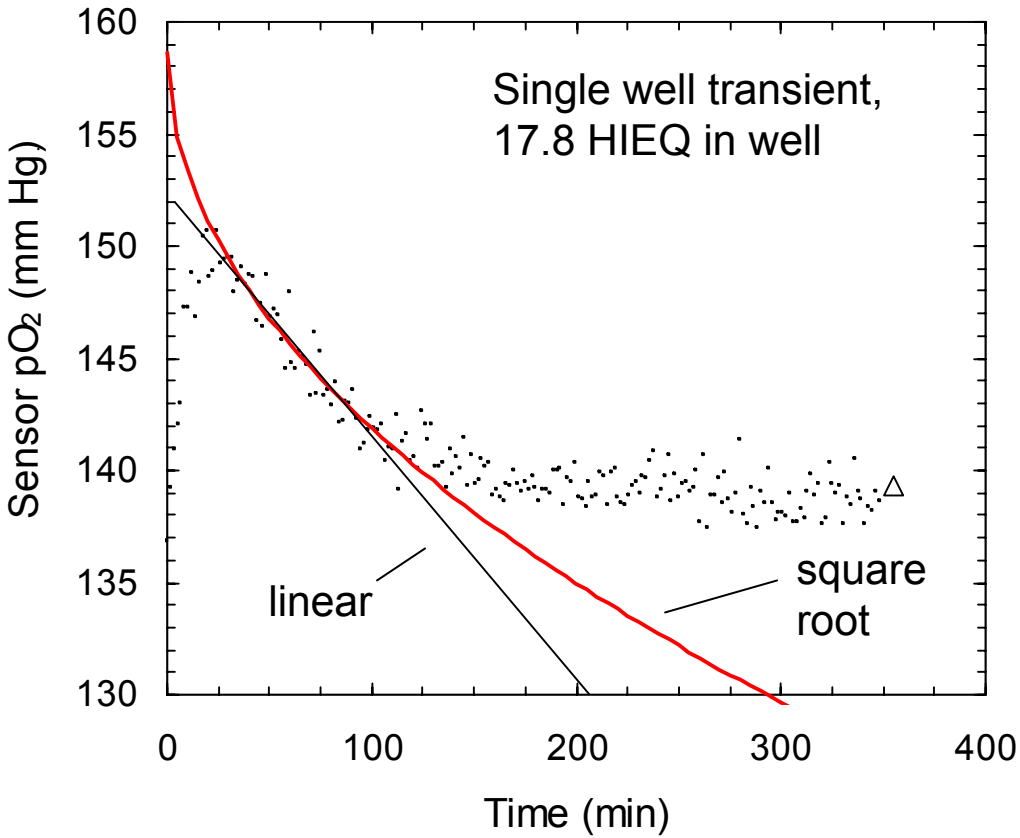
Transient Sensor pO_2 Response

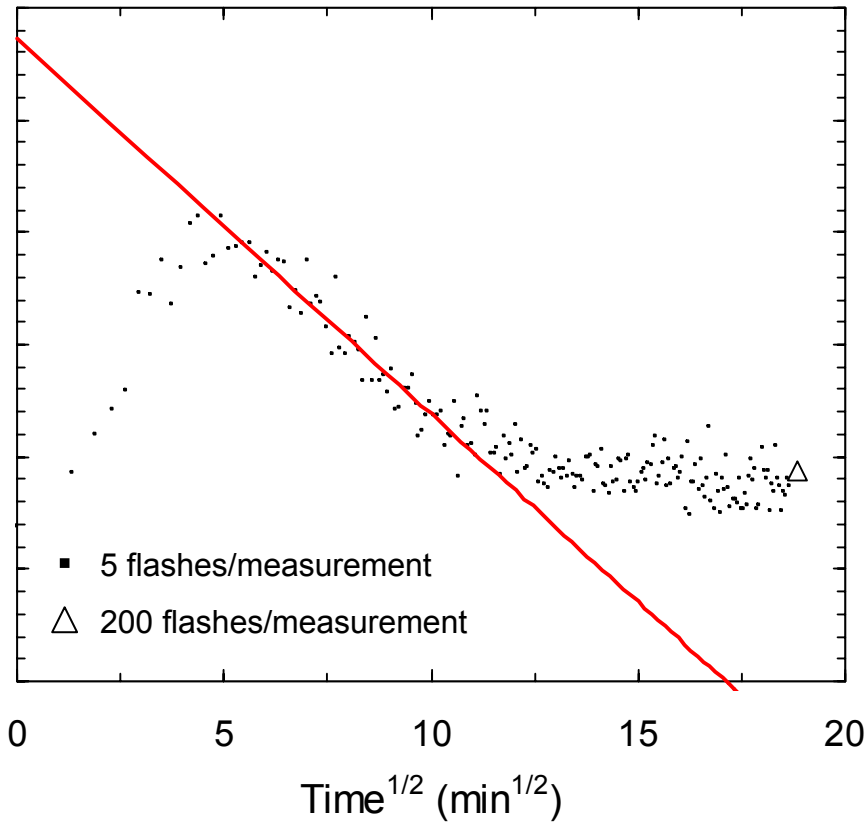
Step change from 0 to 160 mm Hg, no cells present



Immobilization of water with 0.5% agarose dramatically reduces the rate of O_2 transport

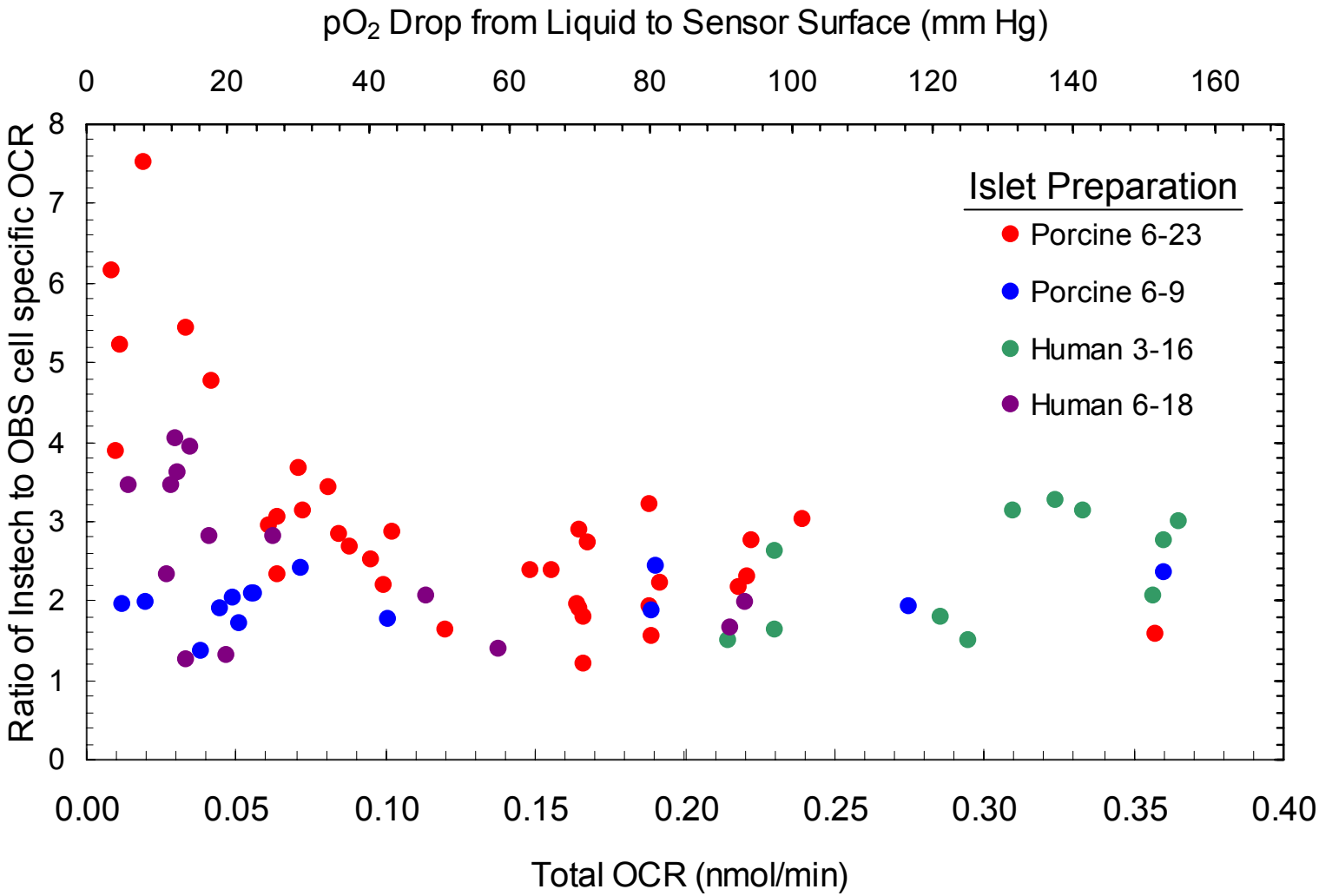
Comparison of OCR Calculated Various ways using the BD OBS





	BD OBS			
	Stirred Tank	Steady State	$T^{1/2}$ fit	T fit
OCR (fmol/min cell)	2.8	1.4	0.71	0.36

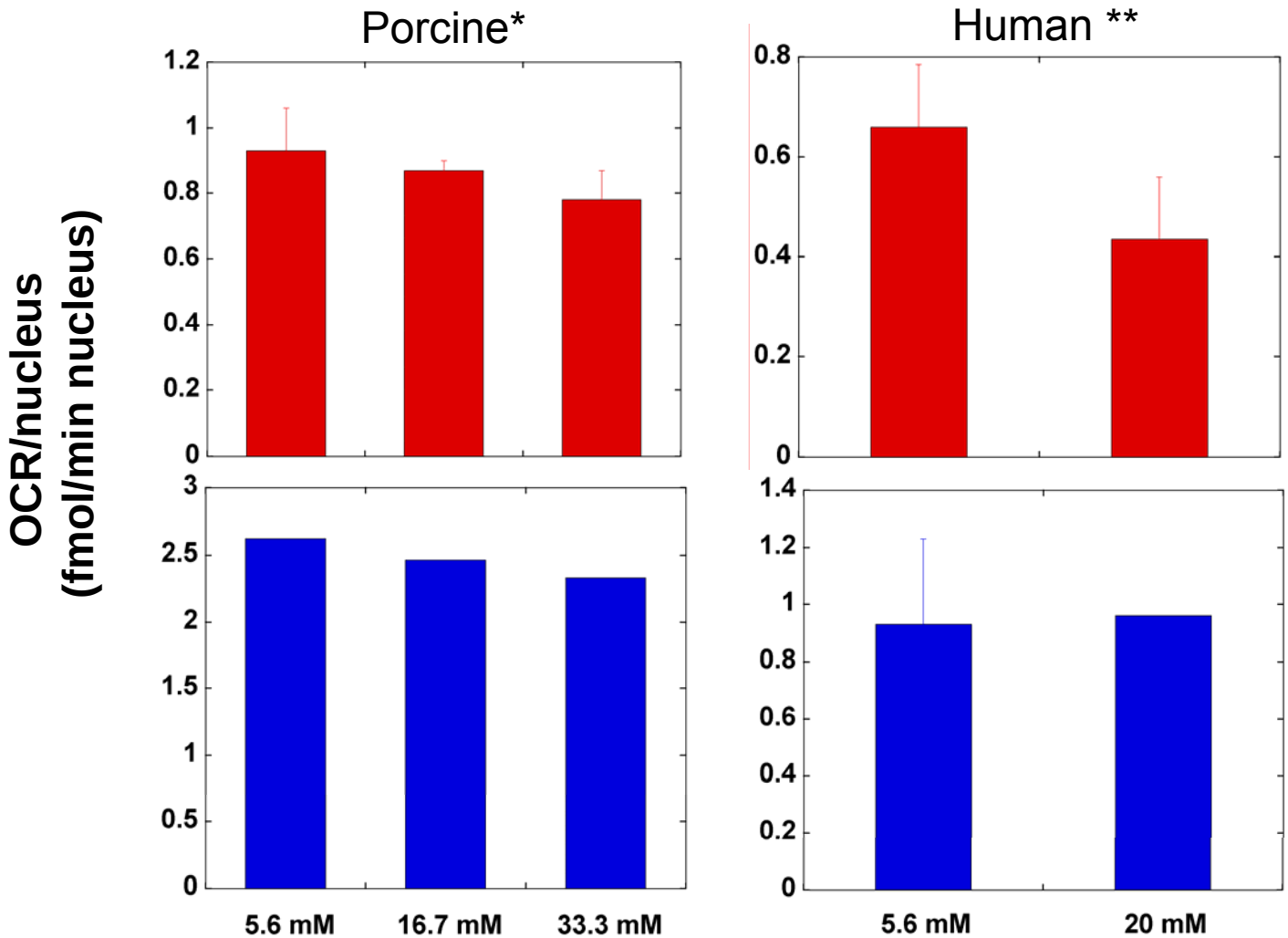
Comparison of OCR/cell measured with Stirred Tank and BD OBS at Different Total OCR values



OCR estimated from steady-state pO_2 reading for all runs shown

Effect of Glucose Concentration on Islet OCR

All measurements in CMRL, one batch of islets, 5 wells
*Procedure of Guarino et al. (2004)
**Continuous reading (20 min)



BD OBS

Instech
Stirred Chamber

No OCR stimulation was observed at any glucose concentration in CMRL

Major Findings with BD OBS

1. Plate reader-induced mixing leads to high sensor pO_2 and lower estimated OCR.
2. High solubility of polystyrene walls and silicone rubber causes long transient period.
3. Additional error is incurred by use of a round well instead of a flat well.

Requirements for Accurate OBS Results

Operating conditions, design, and materials that lead to significant non-ideal conditions should be eliminated

1. Mixing of the liquid in the plate must be avoided
    - movement in the plate reader
    - transport from incubator to plate reader
    - use of agarose may be beneficial
2. If transient data are to be employed, walls should be made of material with much lower O_2 permeability than polystyrene, and the volume of silicone rubber must be reduced. Otherwise, sufficient time must be allotted for the system to reach steady state (quasi-steady state if cells grow).
3. The well geometry should be flat.
4. Sensor pO_2 must be high enough, and/or cell loading must be low enough, so that islet cells do not become oxygen starved

Summary

1. Improvement in islet quality assessment requires development of meaningful, quantitative assays.
2. Nuclei counting combined with microscopy has promise for accurate enumeration of islets.
3. Oxygen consumption rate, which is a measure of oxidative phosphorylation, is a direct measurement of mitochondrial function.
4. OCR measurements made with a stirred chamber using the most purified human islet fraction are predictive of transplantation outcome in mice.
5. The BD OBS is attractive because of its apparent simplicity, but further improvements are needed to ensure meaningful data.

Acknowledgements

MIT/University of Minnesota
Klearchos K. Papas

MIT
Anna Pisania
Daryl E. Powers
Michael J. Rappel
Haiyan Wu
Efstathios S. Avgoustiniatos
Amy S. Lewis

Massachusetts General Hospital
Maria Koulmanda
Hugh Auchincloss
Andy Kipo

University of Minnesota
Bernhard J. Hering

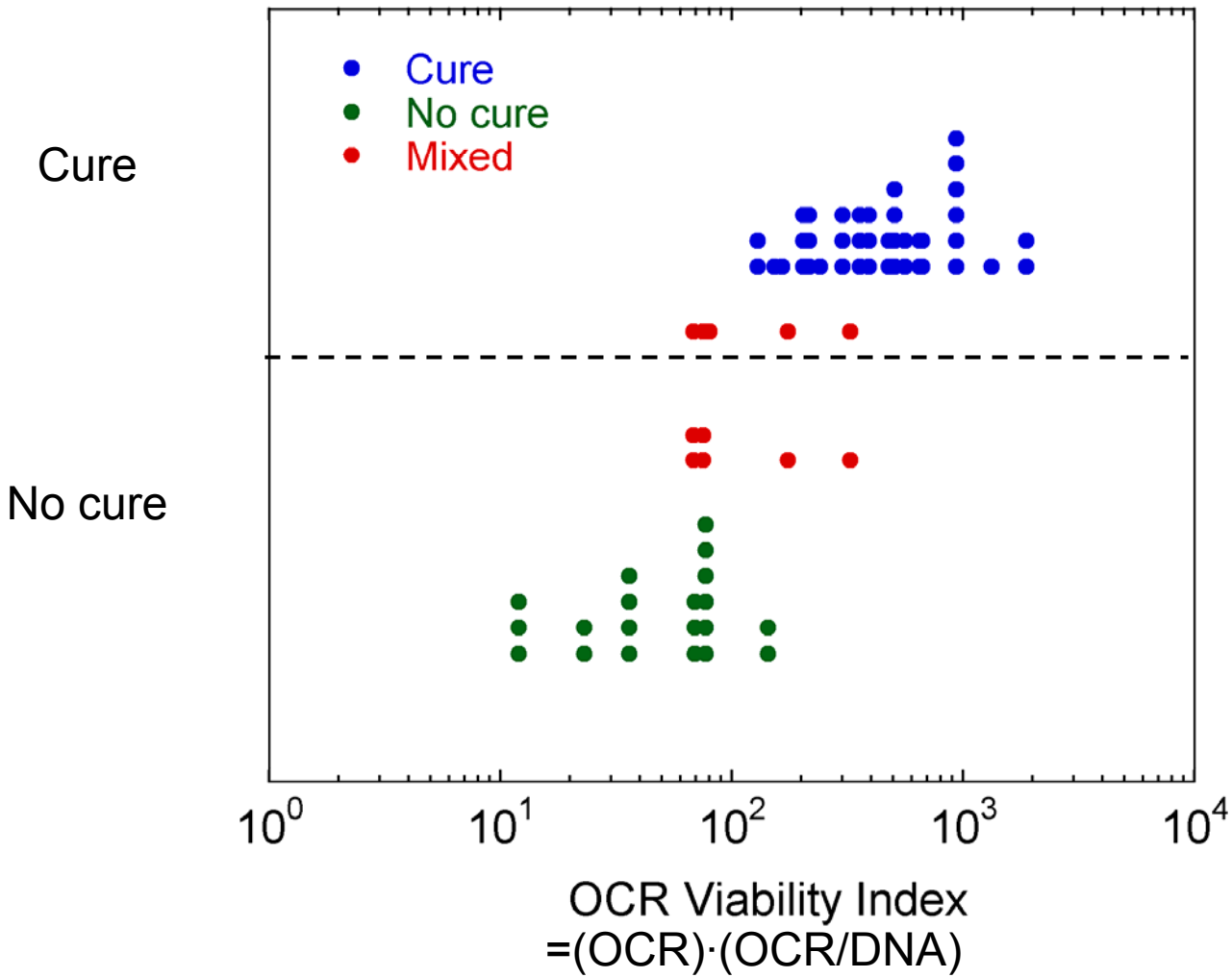
Joslin Diabetes Center
Susan Bonner-Weir
Gordon Weir
Abdulkadir Omer
Vaja Tchipasvilli
Gaurav Chandra
Christopher Cahill

Becton Dickson
Mark Timmins

NIH Grants:
1 R43-DK063727-01 Prodyne
R01-DK063108-01A1
NCRR ICR U4Z 16606
NIDDK SBIR Contract
N44-DK-3-2535 Giner, Inc
JDRF Center for Islet Transplantation
at Harvard Medical School

Extra Slides

Response to Rat Islet Transplants in Diabetic Balb/C Mice (Anti-CD4)



Response to Human Islet Transplants in Diabetic Immunodeficient Mice

Human islets were taken from the highest purity fraction (>90% by DTZ)