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TITLE:

Using an Extracellular Flux Analyzer to Measure Changes in Glycolysis and Oxidative Phosphorylation during Mouse Sperm Capacitation

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SUMMARY:

We describe the application of an extracellular flux analyzer to monitor real-time changes in glycolysis and oxidative phosphorylation during mouse sperm capacitation.

ABSTRACT:

Mammalian sperm acquire fertilization capacity in the female reproductive tract in a process known as capacitation. Capacitation-associated processes require energy. There remains an ongoing debate about the sources generating the ATP which fuels sperm progressive motility, capacitation, hyperactivation, and acrosome reaction. Here, we describe the application of an extracellular flux analyzer as a tool to analyze changes in energy metabolism during mouse sperm capacitation. Using H^+- and O_2- sensitive fluorophores, this method allows monitoring glycolysis and oxidative phosphorylation in real-time in non-capacitated versus capacitating sperm. Using this assay in the presence of different energy substrates and/or pharmacological activators and/or inhibitors can provide important insights into the contribution of different metabolic pathways and the intersection between signaling cascades and metabolism during sperm capacitation.

INTRODUCTION:

The application of mass spectrometry has revolutionized the study of metabolism. Targeted metabolic profiling and metabolomic tracing allow precise monitoring of changes in energy metabolism. However, performing metabolomics successfully requires extensive training, experienced staff, and expensive, highly sensitive mass spectrometers not readily available to every laboratory. In recent years, using an extracellular flux analyzer, such as the Seahorse XFe96 has grown popular as a surrogate method for measuring changes in energy metabolism in various cell types[1-5].

Sperm are highly specialized motile cells; whose task is to deliver the paternal genome to the oocyte. Sperm leaving the male reproductive tract after ejaculation are still functionally immature and cannot fertilize the oocyte because they are unable to penetrate the oocytes` vestments. Sperm acquire fertilization competence as they transit through the female reproductive tract in a maturation process known as capacitation[6,7]. Freshly ejaculated sperm or sperm dissected from the cauda epididymis can be capacitated in vitro by incubation in defined capacitation media containing Ca^{2+}, bicarbonate (HCO_3^-) or a cell-permeable cAMP analog (e.g., dibutyryl-cAMP), a cholesterol acceptor (e.g., bovine serum albumin, BSA), and an energy source (e.g., glucose). During capacitation, sperm modify their motility pattern into an asymmetric flagellar beat, representing a swimming mode called hyperactivation[8,9], and they become competent to undergo the acrosome reaction[7], where proteolytic enzymes are released that digest the oocytes` vestments. These processes require energy, and similar to somatic cells, sperm generate ATP and other high energy compounds via glycolysis as well as mitochondrial TCA cycle and oxidative phosphorylation (oxphos)[10]. While multiple studies demonstrate that glycolysis is necessary and sufficient to support sperm capacitation[11-14], the contribution of oxphos is less clear. Contrary to other cell types where glycolysis is physically coupled to the TCA cycle, sperm are highly compartmentalized and are thought to maintain these processes in separate flagellar compartments: the midpiece concentrates the mitochondrial machinery, whereas the key enzymes of glycolysis appear to be restricted to the principal piece[15-16]. This compartmentalization results in an ongoing debate about whether pyruvate produced in the principal piece by glycolysis can support mitochondrial oxphos in the midpiece, and whether ATP produced by oxphos in the midpiece would be able to diffuse sufficiently rapidly along the length of the flagellum to support the energy requirements in distal parts of the principal piece[17-19]. There is also support of a role for oxphos in sperm capacitation. Not only is oxphos more energetically favorable than glycolysis, generating 16 times more ATP than glycolysis, but midpiece volume and mitochondrial content are directly correlated with reproductive fitness in mammalian species which exhibit greater degrees of competition between males for mates[20]. Addressing these questions requires methods for examining the relative contributions of glycolysis and oxphos during sperm capacitation.

Tourmente et al. applied a 24-well extracellular flux analyzer to compare the energy metabolism of closely related mouse species with significantly different sperm performance parameters[21]. Instead of reporting the basal ECAR and OCR values of non-capacitated sperm, here, we adapt their method using a 96-well extracellular flux analyzer to monitor changes in energy metabolism during mouse sperm capacitation in real-time. We developed a method that allows simultaneously monitoring glycolysis and oxphos in real-time in sperm with beating flagella in up to twelve different experimental conditions by measuring the flux of oxygen (O_2) and protons (H^+) (**Figure 1A**). Due to the breakdown of pyruvate to lactate during glycolysis and the production of CO_2 via the TCA-cycle, non-capacitated and capacitated sperm extrude H^+ into the assay media which are detected by the extracellular flux analyzer via H^+-sensitive fluorophores immobilized to the probe tip of a sensor cartridge. In parallel, O_2 consumption by oxidative phosphorylation is detected via O_2-sensitive fluorophores immobilized to the same probe tip (**Figure 1B**). Effective detection of the released H^+ and consumed O_2 requires a modified sperm buffer with low buffering capacity without bicarbonate or phenol red. Thus, to induce capacitation in the absence

of bicarbonate, we adopted the use of a cell-permeable cAMP analog injected together with the broad-range PDE inhibitor IBMX[22]. Three additional independent injection ports allow the injection of pharmacological activators and/or inhibitors, which facilitates real-time detection of changes in cellular respiration and glycolysis rate due to experimental manipulation.

PROTOCOL:

Sperm are collected from 8-16-week-old CD-1 male mice. Animal experiments were approved by Weill Cornell Medicine's Institutional Animal Care and Use Committee (IACUC).

1. Day prior to assay

1.1. Preparation of sensor cartridge and extracellular flux analyzer calibrant

1.1.1. To hydrate the sensor cartridge, remove the sensor cartridge from the XFe96 Extracellular Flux Assay Kit and place the sensor cartridge upside down next to the utility plate.

1.1.2. Fill a solution reservoir with 25 mL of double-distilled H_2O using a multichannel pipette, add 200 µL of H_2O to each well of the utility plate. Place the sensor cartridge back into the utility plate and incubate overnight in a 37 °C non-CO_2 incubator.

NOTE: Cartridge needs to be hydrated for at least 4 h.

1.1.3. Aliquot 25 mL of the extracellular flux analyzer calibrant into a 50 mL conical tube and incubate it overnight in a 37 °C non-CO_2 incubator.

1.2. Preparation of ConA-coated microplates

1.2.1. Dissolve 2.5 mg of ConA in 5 mL of double-distilled H_2O to prepare a 0.5 mg/mL (w/v) stock.

1.2.2. Using a multichannel pipette, fill each well of an extracellular flux analyzer 96-well plate with 50 µL of the 0.5 mg/mL Con A solution. Leave the lid open and let the plate dry overnight at room temperature.

NOTE: Multiple plates can be coated at once and stored at 4 °C for up to four weeks until ready for use.

1.3. **Preparation of sperm buffer.** Prepare 250 mL of extracellular flux analyzer TYH buffer with **low HEPES** containing 138 mM NaCl, 4.7 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5.6 mM glucose, and 1 mM HEPES. Heat the buffer to 37 °C and adjust the pH to 7.4.

2. Day of the assay

2.1. **Preparation for cartridge calibration.** Replace the H_2O in the utility plate with 200 µL of XF calibrant per well and incubate for at least 1 h in a non-CO_2 37 °C incubator.

NOTE: Instead of calibrant, sterile-filtered PBS, pH 7.4 can be used.

2.2. Heat 50 mL of TYH buffer at 37 °C.

2.3. Generating a wave template for the extracellular flux analyzer

2.3.1. Turn the extracellular flux analyzer on and allow the temperature to stabilize to 37 °C.

2.3.2. Open the wave software and design a new template by opening a blank template (see **Supplemental File 1**, wave template).

2.3.3. Open the **Group definitions** tab. Define assay media as TYH; pH 7.4 and cell type as mouse sperm, leave injection strategies and pretreatments blank. Create different groups for each assay condition; in this example, there are 6 different groups (TYH, TYH + db-cAMP/IBMX, 2-DG, 2-DG + db-cAMP/IBMX, Ant/Rot, Ant/Rot + db-cAMP/IBMX); dibutyryl cAMP (db-cAMP) and 3-Isobutyl-1-methylxanthine (IBMX) to induce capacitation, 2-deoxyglucose (2-DG) as glycolysis inhibitor, antimycin A (antA) and rotenone (rot) as inhibitor of complex III and complex I of the electron transport chain.

NOTE: To account for variability between wells, at least 7-8 wells per condition should be measured in parallel.

2.3.4. Open the **Plate map** tab and define the plate map by assigning groups to specific wells.

NOTE: The four corner wells (A1, A12, H1, H12) will be filled with TYH buffer (no sperm) and will later be used for background subtraction.

2.3.5. Open the **Protocol** tab, add 4 different measurement cycles, select the respective port and edit the measurement details (**Figure 2**, **Table 1**). Highlight *measure after injection* and do not highlight *equilibrate*.

2.3.6. Save the assay template, fill out the project summary and define the saving location for the results file.

2.4. Preparation of compounds to load into sensor cartridge

2.4.1. Prepare 2 mL of 50 mM db-cAMP by dissolving 9.8 mg of compound in TYH buffer and add IBMX to a final concentration of 5 mM.

NOTE: IBMX has the tendency to precipitate after being diluted in TYH buffer. Precipitates can be dissolved by vortexing and incubating the TYH/db-cAMP/IBMX solution in a 37 °C heat block for 5 min.

2.4.2. Prepare 1 mL of 500 mM 2-DG by dissolving 82.1 mg of compound in TYH buffer.

2.4.3. Prepare 1 mL of 5 µM of AntA/Rot by diluting both drugs in TYH buffer.

NOTE: Compounds are diluted 10-fold by injection into the extracellular flux analyzer cartridge; therefore, be sure that injection solutions are 10x more concentrated.

2.5. Isolation of mouse sperm

2.5.1. Anesthetize 3 male mice between 8 and 16 weeks by isoflurane and sacrifice the mice by cervical dislocation after no response to toe pinching was detected. Remove the cauda epididymides and vasa deferentia.

2.5.2. Place each cauda in 500 µL of prewarmed TYH buffer in 24-well plate well, immobilize the cauda to the bottom of the plate using a pair of forceps and open the tissue by making 5-7 small incisions with feather scissors.

2.5.3. Transfer the plate immediately into a non-CO_2 37 °C incubator and let the sperm disperse for 15 min.

2.6. Loading of the sensor cartridge

2.6.1. Two port-loading guides for port A and D and port B and C are provided in the Extracellular Flux kit. For loading a specific port, remove the lid from the sensor cartridge and align the letter of the respective port loading guide with the upper left corner of the cartridge. While injecting, use the fingertips of the non-injecting hand to hold the port loading guide in place and insert the pipette tips vertically into the port loading guide holes.

2.6.2. Port A: Using a multichannel pipette, inject 20 µL of the TYH buffer into every column.

2.6.3. Port B: Inject 22 µL of TYH buffer into column 1-4, inject 22 µL of 500 mM 2-DG into column 5-8, and 25 µL of 5 µM AntA/Rot into column 9-12.

2.6.4. Port C: Inject 25 µL of TYH buffer into every column with odd numbers, inject 25 µL of 10 mM db-cAMP/ 500 µM IBMX into every column with even numbers.

2.6.5. Place the sensor cartridge with the calibration plate into the extracellular flux analyzer and start the assay. Calibration takes 10 – 15 min.

2.7. Preparation of sperm plates

2.7.1. Dissolve 45 mg of BSA in 15 mL of TYH buffer (3 mg/mL w/v) and prepare three aliquots of 3 mL BSA/TYH solution each in 5 mL centrifuge tubes.

2.7.2. Combine two sperm wells each in one 1.5 mL centrifuge tube, count the sperm using a hematocytometer and dilute the sperm to a concentration of 2 x 10^7 sperm/mL.

NOTE: Use cut pipette tips for pipetting sperm to avoid damaging the cells.

2.7.3. Centrifuge the sperm for 3 min at 700 x *g*, remove the supernatant and add 1 mL of TYH buffer. Repeat the centrifugation step, remove the supernatant, and transfer each sperm suspension aliquot into one 5 mL centrifuge tube with 3 mL of BSA/TYH.

2.7.4. Place 180 µL of TYH buffer into each corner well (A1, A12, H1, H12) of a ConA-coated plate. Place 180 µL of sperm suspension into each empty well of the ConA-coated plate (1.2 x 10^6 sperm/well).

2.7.5. Centrifuge the sperm plate at 250 x *g* for 1 min, rotate the plate by 180°, and centrifuge again at 250 x *g* for 1 min (lowest braking rate 1).

2.7.6. Remove the calibration plate from the extracellular flux analyzer and add the sperm plate. Continue the assay.

2.8. Data extraction and analysis

2.8.1. After finishing the assay, remove the cartridge, open the results file, click the ***Export*** tab and export the completed run as a GraphPad Prism file (**Supplemental File 2**: Primary example data used for **Figure 3**).

2.8.2. Duplicate the ECAR and OCR file by clicking on the ***New*** tab and then the ***Duplicate Family…*** tab (**Figure 3A**).

2.8.3. Delete the first 7 rows of data (**Figure 3B**).

2.8.4. Normalize to the data point before the cAMP/IBMX injection by baseline subtracting row 1. Click on the **Analyze** tab, then on the **Remove baseline and column math** tab. Highlight *Selected row(s)*: ***First Row***, ***Calculation***: ***Ratio:Value/Baseline***, and ***Subcolumns***: ***Ignore Subcolumn***. Click **OK** (**Figure 3C**).

REPRESENTATIVE RESULTS:

This method uses an extracellular flux analyzer to monitor real-time changes in the rate of glycolysis and oxphos during mouse sperm capacitation. **Figure 4** shows an exemplary experiment where sperm were capacitated in the presence of glucose as the only energy substrate and 2-DG and antimycin and rotenone as pharmacological modulators. The energy

substrate in the extracellular flux analyzer TYH buffer and the pharmacological modulators can be freely selected depending on the goal of the experiment. Non-capacitated mouse sperm in BSA/TYH were attached to the bottom of a ConA-coated transient microchamber via their head. In this example, basal ECAR and OCR values on average between all the detected wells were 12.76 ± 2.75 mpH/min and 23.64 ± 2.78 pmol/min, respectively.

After a mock injection with TYH buffer, followed by injection of 2-DG and ant/rot to inhibit glycolysis and oxidative phosphorylation, respectively, sperm capacitation was induced by injection of db-cAMP/IBMX.

The representative results show that in the presence of glucose, capacitation is accompanied by a 7-fold increase in Extracellular Acidification Rate (ECAR), which is inhibited by blocking glycolysis with 2-DG (**Figure 4A**). Capacitated sperm show a 20-fold increase in Oxygen Consumption Rate (OCR) compared to non-capacitated sperm (**Figure 4B**), demonstrating that mouse sperm enhance both glycolysis and oxidative phosphorylation to support the increasing energy demand during capacitation. The rise in ECAR during sperm capacitation is inhibited by the glycolysis inhibitor 2-DG, but not affected by the oxidative phosphorylation inhibitors antimycin A and rotenone (**Figure 4C**), indicating that the change in ECAR is mainly driven by H^+ release from glycolysis. The increase in OCR is, as expected, blocked by antimycin A and rotenone (**Figure 4D**), but it is also inhibited by 2-DG (**Figure 4B**) revealing that the increase in oxphos during sperm capacitation is dependent on glycolytic activity.

FIGURE AND TABLE LEGENDS:

Figure 1: Principle of the extracellular flux analyzer. (A) Due to the breakdown of glucose to lactate during glycolysis and the generation of CO_2 via the TCA cycle, changes in glycolysis and oxphos are accompanied by H^+ excretion into the extracellular media. The XFe96 Analyzer detects these changes in extracellular H^+ concentration as ECAR. In parallel, changes in extracellular O_2 concentration due to O_2 consumption by oxidative phosphorylation is measured as OCR. Blocking glycolysis with 2-deoxyglucose (2-DG) or respiration with the complex I and complex III inhibitors rotenone and antimycin A reveals which metabolic pathways support the increasing energy demand during sperm capacitation. **(B)** Mouse sperm are attached via their heads to the bottom of a ConA-coated microchamber; their flagella are freely moving. While changes in the extracellular H^+ and O_2 concentration are detected by H^+- and O_2-sensitive fluorophores immobilized to a sensor probe, up to four different compounds can be injected sequentially.

Figure 2: Schematic representation of the exemplary experiment. Changes in ECAR (mpH/min) and OCR (pmol O_2/min) are detected in non-capacitated and capacitating sperm using an extracellular flux analyzer. Cycle 1: Basal ECAR and OCR values. Cycles 2-5: System stabilization after TYH mock injection. Cycles 6-8: Drug incubation. Cycles 9-27: Sperm capacitation. Arrows indicate injections. 2-DG: final concentration 50 mM, AntA/Rot: final concentration 0.5 µM, db-cAMP: final concentration 1 mM, IBMX: final concentration 500 µM.

Figure 3: Data analysis. (A) Raw data of changes in ECAR during mouse sperm capacitation. **(B)** Data after removal of the first 7 data points. **(C)** Data normalized to the data point before

cAMP/IBMX injection. Data is shown as mean of 7-8 wells ± S.E.M. Injections are indicated with an arrow.

Figure 4: Changes in glycolysis and oxidative phosphorylation during mouse sperm capacitation. (A) Normalized ECAR in non-capacitated and capacitating mouse sperm in the presence and absence of 50 mM 2-DG. **(B)** Normalized OCR in non-capacitated and capacitating mouse sperm in the presence and absence of 50 mM 2-DG. **(C)** Normalized ECAR in non-capacitated and capacitating mouse sperm in the presence and absence of 0.5 µM antimycin A and rotenone. **(D)** Normalized OCR in non-capacitated and capacitating mouse sperm in the presence and absence of 0.5 µM antimycin A and rotenone. Data is shown as mean ± S.E.M normalized to the data point before db-cAMP/IBMX injection; n = 6. Injections are indicated with an arrow.

Table 1: Measurement Details

Supplemental Figure 1: Capacitation of mouse sperm in extracellular flux analyzer TYH buffer. Phosphorylation of tyrosine residues of mouse sperm detected at different time points during capacitation (0 – 90 min) after incubation in **(A)** TYH with 25 mM HCO_3^-, 3 mg/mL BSA and 20 mM HEPES or in **(B)** Extracellular flux analyzer TYH with 5 mM db-cAMP, 500 µM IBMX, and 1 mM HEPES, detected with an α-phosphotyrosine antibody.

Supplemental file 1: Wave assay template to detect changes in glycolysis and oxidative phosphorylation during mouse sperm capacitation. The wave desktop software can be downloaded for free after filling out a registration form (www.agilent.com/en/products/cell-analysis/cell-analysis-software/data-analysis/wave-desktop-2-6) and installed on windows 7, 8 or 10, Mac OSx 10.11 (or higher) with Parallels 12 (or higher). Thereby, wave templates can be generated independently from the extracellular flux analyzer, exported and then imported into the wave software of any extracellular flux analyzer.

Supplemental file 2: Graph pad prism file exported from wave software with exemplary data analysis.

DISCUSSION:

The loss of sperm capacitation in the absence of certain metabolic substrates or critical metabolic enzymes revealed energy metabolism as a key factor supporting successful fertilization. A metabolic switch during cell activation is a well-established concept in other cell types, however, we are just beginning to understand how sperm adapt their metabolism to the increasing energy demand during capacitation. Using an extracellular flux analyzer, we developed an easily applicable tool to monitor changes in glycolysis and oxidative phosphorylation in real-time during sperm capacitation. The detection of changes in extracellular H^+ and O_2 with fluorophores immobilized to a sensor probe is minimally invasive and the four individually operated injection ports allow manipulation with pharmacological inhibitors or activators at distinct time points before or during the capacitation process. This protocol gives only one example of a mouse sperm capacitation experiment. To simplify the interpretation of the results we chose the show an

exemplary experiment where glucose was used as the only energy source. The conditions are variable depending on the goal of the experiment, and up to 12 different conditions (i.e., different energy sources like glucose vs. glucose and pyruvate) can be measured in parallel. Additionally, four independent injection ports allow the injection of pharmacological activators and/or inhibitors at any desired time point before or during capacitation. This opens the possibility to use the extracellular flux analyzer as a semi high-throughput screening device. Similar to mouse sperm, in other species like human or bovine it is still enigmatic how sperm change their metabolism during capacitation. The protocol can be easily adapted; thus, we recommend optimizing the sperm concentration each time before starting a real experiment.

The protocol´s biggest limitation is that high-quality results can only be achieved in the absence of bicarbonate. Bicarbonate in seminal fluid is the physiological signal that initiates the sperm's capacitation signaling cascade following ejaculation. Bicarbonate activates the soluble adenylyl cyclase (sAC; ADCY10), which catalyzes the conversion of ATP into cAMP[23]. The increase in cAMP then drives a signaling cascade mediated by Protein Kinase A, which ultimately leads to downstream tyrosine phosphorylation of target proteins (e.g., ion channels, metabolic enzymes, and structural proteins[24,25]). This restriction against bicarbonate is overcome by injecting the product of bicarbonate-activated sAC, cAMP. We use 5 mM of the cell-permeable cAMP analog db-cAMP in parallel with the broad-specificity phosphodiesterase inhibitor IBMX, which prevents rapid degradation of db-cAMP by phosphodiesterases. This combination effectively initiates the cAMP-regulated capacitation signaling pathway post sAC activation with a similar kinetic as bicarbonate (**Supplementary Figure 1**). In parallel to bicarbonate, a cholesterol acceptor (e.g., BSA) is used to in vitro capacitate freshly ejaculated sperm or sperm dissected from the cauda epididymis. Albumin cannot be injected because it clogs the injection port and, therefore, needs to be added to the sperm buffer before plating the cells. Performing the experiment in the presence or absence of BSA revealed that the increase in ECAR and OCR during sperm capacitation is independent of the cholesterol acceptor. However, the presence of BSA in the sperm buffer decreased fluctuations in the detected ECAR and OCR values between different wells and experiments; thus, we highly recommend including BSA in the sperm buffer to increase reproducibility.

Isolating sperm from the cauda epididymis results in the contamination of sperm with epididymal fluid. To avoid artificial results due to seminal fluid components, we recommend washing sperm two times before using them for an experiment. Sperm concentration and plating is another critical factor determining the success of the experiment. For reliable results, the manufacturer recommends initial ECAR values to be larger than 10 and OCR values to be larger than 20. The sperm concentration used in this protocol was optimized so that the average basal ECAR and OCR values of the 7-8 wells measured per condition are above 10 and 20, respectively. Freely moving sperm disturb the detection of changes in extracellular H^+ and O_2. Thus, it is crucial to adhere all sperm with their head to the bottom of the plate. We found success adhering sperm by coating the plate with ConA, a plant lectin that specifically interacts with the outer acrosomal membrane and is commonly used for acrosome assays[26], and by gently spinning the plate (see step 2.7.3). With this method, sperm are localized to the bottom of the well solely via their head so they can still freely move their flagella and change their flagellar beating pattern during capacitation.

Sperm constantly extrude H^+ and O_2 in both, the non-capacitated and the capacitated state. To determine the initial ECAR and OCR as accurately as possible, it is crucial to start the experiment as quickly as you can after the last washing step. This can be accomplished by loading the sensor cartridge while the sperm are swimming out and by starting the method in the extracellular flux analyzer before the first washing step. Calibrating the instrument takes approximately the same time as washing and plating the cells and spinning the plate. The manufacturer recommends an equilibration phase to allow the system to stabilize before the first real data point is measured. Since the protocol includes 8 measurement cycles before capacitation is initiated, to save time, the equilibration step is excluded from this protocol.

The ability to inject solutions during the assay and to observe their effects on respiration and glycolytic rate in real-time is a key feature of the extracellular flux analyzer. Loading the sensor cartridge is one of the critical steps in the protocol and should be carried out carefully. To ensure proper injection into all wells, each series of ports needs to contain the same volume, including the background wells. Loading the ports with a multichannel pipette requires some practice but decreases variability and loading time considerably. We highly recommend using the port loading guide but to inject only four ports simultaneously. It is also important to appreciate that during loading, the injection volumes are gradually increased to compensate for the increasing volume in the well. While loading the sensor cartridge, it is important to not fully insert the tips into the port. This might prematurely push injection solution through the port orifice. While establishing the method we found that injecting liquid into a sperm well causes unwelcome injection artifacts, probably due to dilution of the sperm in the well and/or displacing sperm from the well bottom. The first injection causes the largest injection artifact, so we included a mock injection with sperm buffer into all wells at the beginning of the protocol.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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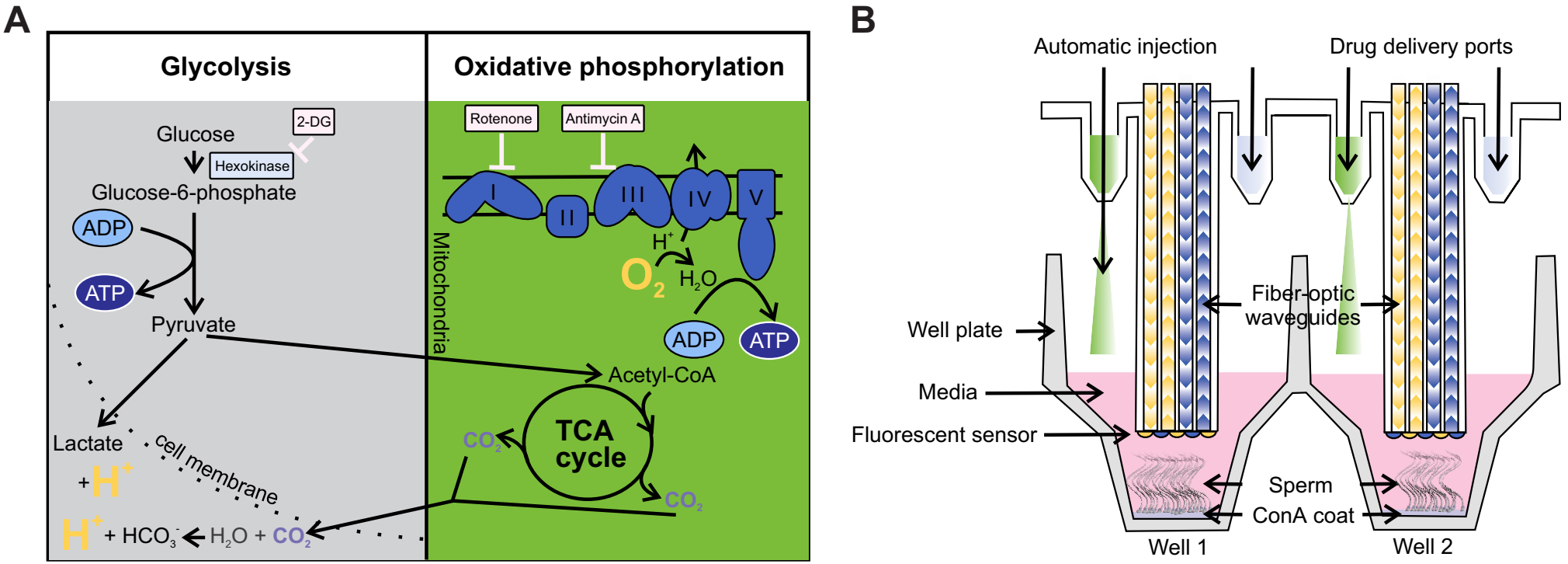

Figure 2

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Figure 3

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Figure 4

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Figure S1

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Figure S1.svg

Table

	Port	Number of cycles	Mix (min)	Wait (min)	Measure (min)
A: Basal ECAR/OCR	no port	1	2:00	0:00	3:00
B: Mock injection	1	4	2:00	0:00	3:00
C: Drug injection	2	3	2:00	0:00	3:00
D: Capacitation	3	18	2:00	0:00	3:00

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Reagents			
2-Deoxy-D-glucose	Sigma-Aldrich	D8375	2-DG
3-Isobutyl-1-methylxanthine	Sigma-Aldrich	I7018	IBMX; prepare a 500 mM st
Antimycin A	Sigma-Aldrich	A8674	AntA; prepare a 5 mM stocl
Bovine serum albumin	Sigma-Aldrich	A1470	BSA
Calcium chloride	Sigma-Aldrich	C1016	CaCl2
Concanacalin A, Lectin from Arachis hypogaea (peanut)	Sigma-Aldrich	L7381	ConA
Glucose	Sigma-Aldrich	G7528	
Hepes	Sigma-Aldrich	H0887	
Isothesia	Henry Schein Animal Health	1169567761	Isoflurane
Magnesium sulfate	Sigma-Aldrich	M2643	MgSO4
N6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt	Sigma-Aldrich	D0627	db-cAMP
Potassium chloride	Sigma-Aldrich	P9333	KCl
Potassium dihydrogen phosphate	Sigma-Aldrich	P5655	KH2PO4
Rotenone	Cayman Chemical Company	13995	Rot; prepare a 5 mM stock
Sodium bicarbonate	Sigma-Aldrich	S5761	NaHCO3-
Sodium chloride	Sigma-Aldrich	S9888	NaCl
Equipment and materials			
12 channel pipette 10-100 µL	eppendorf	ES-12-100	
12 channel pipette 50-300 µL	vwr	613-5257	
37 °C, non-CO_2 incubator	vwr	1545	
5 mL cetrifuge tubes	eppendorf	30119380	
50 mL conical centrifuge tubes	vwr	76211-286	
Centrifuge with plate adapter	Thermo Scientific	IEC FL40R	
Dissection kit	World Precision Instruments	MOUSEKIT	

Inverted phase contrast microscope with 40X objective	Nikon		
OctaPool Solution Reservoirs, 25 ml, divided	Thomas Scientific	1159X93	
OctaPool Solution Reservoirs, 25 mL, divided	Thomas Scientific	1159X95	
Seahorse XFe96 Analyzer	Agilent		
Seahorse XFe96 FluxPak	Agilent	102416-100	Also sold as XFe96 FluxPa

tock solution in DMSO (111.1 mg/ml) and store in small aliquots
k solution in DMSO (2.7 mg/ml) and store in small aliquots

solution in DMSO (2mg/ml) and store in small aliquots

k mini (102601-100) with 6 instead of 18 cartidges.

Editorial comments:
The manuscript has been modified and the updated manuscript, **60815_R0.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

We revised the manuscript according to the suggestions of the reviewers. Responses to the reviewer's comments are added in cursive.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

After revising the manuscript, we proofread the text for grammar and spelling errors.

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These figures have not yet been published elsewhere; can we reserve the right to publish them elsewhere or delay publication of this JOVE article until after our other manuscript is published?

3. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol steps (including headings and spacing) in yellow that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We amended the protocol section of the manuscript accordingly.

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We removed the brand name Agilent from the manuscript and use the generic term Seahorse XFe96 Analyzer instead.

5. Please specify the anesthesia or euthanasia method.

We specified the anesthesia and euthanasia method (181/182) and added isoflurane to the Jove_Materials excel file.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Good method to determine metabolic energy changes in sperm/

Major Concerns:

None

Minor Concerns:
None

Reviewer #2:
Manuscript Summary:
The SeaHorse system is applied to track energetic metabolism in spermatozoa subjected to capacitation conditions. The system tracks changes in glycolysis and OXPHOS using H+ and O2 sensitive fluorophores. The use of this system is supported by previous publications.

Major Concerns:
The lack of bicarbonate in the media, perhaps necessitates a proper control for capacitation in a separate experiment comparing the cAMP analogue with bicarbonate tracking changes in motility patterns, tyrosine phosphorylation or response of acrosome induced reaction after a Ca2+ ionophore challenge.

We revised the manuscript to include a new supplemental figure demonstrating the efficacy of 5 mM db-cAMP, 500 µM IBMX and 1 mM HEPES in TYH compared to standard capacitation conditions (see fig. S1). These results confirm that 5 mM db-cAMP, 500 µM IBMX and 1 mM HEPES are capable of capacitating sperm consistent with the seminal studies on capacitation published in Visconti et al, 1995, where the authors successfully capacitate mouse sperm using similar conditions (1 mM db-cAMP, 100 µM IBMX, 25 mM HEPES). We added the respective citation (84).

Figures of the different parts that the system has, will facilitate the comprehension of the protocol. As stated in the printed version is hard to follow what part of the system they are talking about. Don know if this is present in additional files, I could not open it.

A schematic representation of the reaction chamber and the Seahorse XFe96 Analyzer sensor cartridge probe tip is shown in Figure 1b. Further information and diagrams are provided by the manufacturer (Agilent), and it would not be appropriate to use the product literature in this manuscript.

Minor Concerns:
Use of validating additional experiments measuring lactate ATP, and capacitation in subsamples subjected to the same conditions will improve the robustness of the assay

The revised manuscript now includes the best-established molecular readout for capacitation (tyrosine phosphorylation), and we feel any further validation would be unnecessary. In fact, methods developed here will enable researchers to validate the previously postulated changes in lactate and ATP during capacitation.

Reviewer #3:
Manuscript Summary:
In this manuscript, Balbach et al. described a detailed protocol of measuring extracellular acidification

rate (ECAR) and oxygen consumption rate (OCR) by seahorse XF Analyzer to monitor changes in glycolysis and oxidative phosphorylation of sperm cells in real-time. This will be a very useful resource/visualization for the unexperienced, potential users in the field. In addition, they show that mouse sperm enhance both glycolysis and oxidative phosphorylation during capacitation, which provides plausible explanation how sperm provide extra energy to maintain the motility when hyperactivated.

Major Concerns:

1. The strategy of ECAR/OCAR measurement used in this paper is different from the standard method using the same analyzer with other cells or the previous publication by Tourmente (2015) where the whole procedure is done in one well continuously. For example, oligomycin, FCCP and antimycin A/Rotentone are added consecutively which gives a visualized readout for OCR. The authors should justify the current design.

Instead of using the Seahorse XFe96 analyzer to report basal ECAR/OCR values of non-capacitated mouse sperm as described in Tourmente et al. we adapted the method so that it allows real-time monitoring of changes in ECAR and OCR during sperm capacitation. This not only provides the ECAR and OCR of capacitated sperm after 90 min of capacitation but also allows observations of changes in ECAR and OCR in real-time during capacitation. Thus, this method is complementary to Tourmente et al. and provides more information than the standard method. We amended the manuscript to make this point more clearly (69 - 75).

2. In this paper, the authors use db-cAMP and phosphodiesterase inhibitor, IBMX, to induce capacitation because of the technical limitation of using the standard capacitation buffer with bicarbonate. Did authors examine the efficiency of cAMP/IBMX on sperm capacitation? For example, how much cells develop hyperactivated motility?

As described above in response to Reviewer #2, we compared the capacitation-induced tyrosine phosphorylation in TYH buffer containing 25 mM HCO_3^-, 3 mg/ml BSA and 20 mM HEPES and Seahorse TYH buffer containing 5 mM db-cAMP, 500 µM IBMX and 1 mM HEPES (see Fig. S1). This experiment confirms the capacitation of mouse sperm under the described experimental conditions.

3. Figure1A, Antimycin A inhibits the flow of electrons through complex III of the ETC by blocking the passage of electrons from cytochrome b to cytochrome c, while Rotenone inhibits the transfer of electrons from iron-sulfur centers in complex I to ubiquinone. The annotation was not displayed correctly.

We thank the reviewer for catching this mistake and modified Fig. 1A and the manuscript accordingly (283-284).

4. Figure1A, H2O+CO2◊H++HCO3-

We thank the reviewer for catching this mistake and modified Fig. 1A accordingly.

5. In the manuscript, the rationale for revised TYH medium was used by removing of bicarbonate and BSA were described. However, the reason why pyruvate was removed from TYH medium was not described?

We agree with the reviewer that studying the contributions of additional carbon sources define interesting experiments, but these studies exceed the scope of this manuscript. In this manuscript, we show one exemplary experiment in the presence of the most simple nutrient conditions supporting capacitation; i.e., glucose alone (see Goodson et al. 2012 as reference). To make the reviewer's point that additional studies would be useful for discovering nutritional requirements during capacitation, we amended the manuscript by discussing how the protocol could be modified (254-256, 341-347).

6. It is not clear whether we can reliable compare ECAR and OCAR measurement from non-capaciated sperm to capacitated cells from Figure 3 and 4, all the injection points should be indicated as in Figure 2 to clarify.

We added arrows to indicate the injection points in Fig. 3 and 4.

7. Did authors capacitate sperm all following 2-DG and AntA/Rot treatment? Have authors ever capacitate sperm before inhibitors application to make it clear that the blockers inhibit the capacitation process or indeed metabolism after capacitation?

We agree with the reviewer that these would be very interesting experiments (i.e., studies which determine the relative contributions of oxphos and glycolysis during and after capacitation), but these studies exceed the scope of this JOVE manuscript describing a new method. In this manuscript, we show a single exemplary measurement where 2-DG and Ant/Rot were added before capacitation was initiated to demonstrate that we are truly measuring glycolysis and oxphos. Similar to point 5 above, we amended the manuscript stating that it is possible to add pharmacological modulators at any time point before or during capacitation (345-347).

Minor Concerns:
1. Line 137, 138: please spell out db-cAMP, 2-DG, Ant/Rot, and their corresponding function. They are used first in main body of manuscript.

We amended the manuscript accordingly.

2. Line 113: Is there a time limit for the coated plate?

We have stored plates up to four weeks and added this information to the protocol.

3. Line131: to 37˚C ---at 37˚C

We amended the manuscript accordingly.

Reviewer #4:
Manuscript Summary:
The manuscript describes a method to measure metabolism in sperm. It addresses some of the issues involved in the approach and provides reasonable solutions. This is a well written manuscript.

Major Concerns:
None

Minor Concerns:
There are other species such as the bovine in which glycolysis inhibits capacitation and may have been of interest to include but could be mention.

We agree such studies would be very interesting, but they would exceed the scope of this JOVE manuscript describing a new method. We included a section in the protocol stating the same protocol can easily be adapted to sperm from other species like bovine or human (348-351).

When you use IBMX to increase cAMP in sperm you often change kinetics of capacitation and this was not mentioned.

To address this point, we compared the time course of capacitation-induced tyrosine phosphorylation in TYH buffer containing 25 mM HCO_3^-, 3 mg/ml BSA and 20 mM HEPES and Seahorse TYH buffer containing 5 mM db-cAMP, 500 µM IBMX and 1 mM HEPES (see Fig. S1). This experiment confirms that the kinetic of capacitation is similar between capacitation induced by HCO_3^- and db-cAMP/IBMX.

Reviewer #5:
The authors present a protocol to assess glycolysis and oxidative phosphorylation in mouse spermatozoa under conditions that presumably promote capacitation, using a Seahorse XFe96 analyzer. Improvements in both the protocol (more work is needed) and in the way it is presented are required.

There are three major issues with the protocol, as presented by the authors:

(a) It is not known how many sperm they have in each well and, therefore, there is no normalization of data using sperm numbers. The procedure for attachment of sperm to the well is unreliable and the authors do not take measures to adjust for differences between wells or between experiments.

(b) It is not known if the sperm are really capacitated. Although they assume that the sperm are undergoing capacitation under the conditions of incubation, they do not show any validation which is important because conditions of incubation in the Seahorse (removal of HCO3 which the authors replace with 1 mM Hepes) are not explored in parallel by using molecular handles of capacitation.

(c) There is no indication of cell viability or vigour of motility during the experiment.

SPECIFIC COMMENTS
The authors quote Tourmente et al (2015) as a previous study in which a Seahorse was used to study sperm metabolism in the mouse, and it seems to me that most of the protocol is actually based on the methods in that paper. I suggest this is acknowledged.

We revised the introduction to more clearly state that the current method is adapted from the method of Tourmente et al. (71-73).

Experimental design is not clear in Fig. 2: it looks as a linear design rather than several alternatives after cycle 5.

We exchanged the '/' in Fig. 2 with 'or' to make clear we inject alternative conditions after cycle 5 and cycle 9.

In addition, their approach is rather peculiar when testing cell responses.... they inhibit glycolysis or oxphos before starting capacitation but they do not test the response of cells to inhibitors at some point in capacitation.

As we responded to Reviewer #4, this manuscript shows a single exemplary measurement where 2-DG and Ant/Rot were added before capacitation was initiated to demonstrate that we are truly measuring glycolysis and oxphos. Further studies would exceed the scope of this JOVE manuscript describing a new method, and we amended the manuscript stating that it is possible to add pharmacological modulators at any time point before or during capacitation (345-347).

A photograph showing sperm attached to the well (or a video showing them moving/beating) should be included. It is important to see what is the degree of homogeneity and density in seeding the authors get with their method.

We are unclear how such a photograph would add to the method as written here. As described below, instead of assuming anything about quantitative seeding, we normalize each well to itself, which provides a more robust and quantitative way to normalize the data. We anticipate a video capture of the wells during videography of the method.

Lines 213-215 (2.7.3): Authors should indicate what is the sperm concentration after these steps.

In 2.7.4. we state that the final sperm concentration per well is 1.2 x 10^6.

Lines 221-222 (2.7.5): Indicate if brake is on (this is important for distribution of cells).

We use the lowest braking rate 1 and added this information to the manuscript (230).

The protocol does not include a step (after the experiment) in which cells are counted and hence there is no normalization for sperm numbers. This is very important and, without doing this, the authors are assuming (wrongly) two crucial things:
(i) That they are placing 1.2 million sperm in each well after 2 washes/resuspensions. This is unrealistic because cells are lost and the concentration will not always be the same and will vary between experiments. When sperm are washed at 250xg there will inevitably be some sperm loss.
(ii) That sperm attachment is homogeneous; that is, that all sperm attach and that they attach to both bottom and walls (with the volume of ConA solution they are using there is likely attachment of sperm to the walls).
So, altogether, a major problem here is that the authors do not know how many sperm they are basing their measurements on.

Methods to normalize sperm numbers at the end of the assay are less accurate than the internal normalization performed in the method described here. For example, classical normalization methods such as cell number counting or quantifying protein or DNA concentration include inherent biases. Because sperm cannot be counted directly in the wells of the 96-well plates used, counting sperm requires removing the sperm from the ConA-coated well bottoms of each well. Not only is this needlessly labor-intensive, but it will be error-prone since sperm recovery from each of the ConA-coated wells will be variable. Similarly,

quantifying sperm content in each well by measuring the concentration of protein or DNA would be biased because it does not distinguish between live and dead sperm. We excluded using assays measuring viable cells, such as MTT or ATP assays, for normalization because they are based on cell metabolic activity, which changes during capacitation and in response to the pharmacological treatments being tested. Instead, we normalize each individual well to the ECAR and OCR value measured at the time point immediately before capacitation is initiated; thus, all data are reported as the fold change in ECAR and OCR of non-capacitated and capacitated sperm. Using this normalization method, the results of the experiment are independent of the cell number and well to well variation is mitigated because each condition has its own baseline.

Supplementary material: please include screenshots for data extraction and analysis rather than than files as included now.

Since the data extraction will be shown in the video we don't think it is necessary to include a screenshot. Instead of showing screenshots of the data analysis we include an actual exported data file so that the reader can reproduce the analysis steps using a widely available graphing software.

Raw OCR data are needed. Only fold change for OCR is given (Fig. 4, whereas raw data and fold change are shown for ECAR. It is very important to see what kind of results authors get with their methods.

We included the averaged basal ECAR and OCR values in the manuscript (258-259).

Line 350: For reliable results, the manufacturer recommends initial ECAR values to be larger than 10 and OCR values to be larger than 20. Clearly this is not the case in the results given so measurements are below error values.

Fig. 3A shows a basal ECAR value of 13.73 ± 2.99 for TYH and 11.79 ± 2.51 for TYH + db/cAMP/IBMX. Although we are aware that the error bar of the second condition is below 10, the mean value is above 10 and therefore in the range indicated by the manufacturer. We amended the manuscript clarifying that we optimized the assay for a basal ECAR and OCR value over 10 and 20 averaged over the ≥7 wells for each condition (377-379).

Line 352: "Freely moving sperm disturb the detection of changes in extracellular H+ and O2." Please justify. It should not be so.

Since the fluorophores are immobilized to the sensor cartridge probe tip, changes in H^+ and O_2 can only be reliably measured if H^+ and O_2 have unlimited access to those fluorophores. Freely moving sperm in the microchamber not only limit the diffusion of H^+ and O_2 in the Seahorse TYH buffer but can also restrict the access to the immobilized fluorophores by blocking the probe tip surface.

Lines 380-383: The authors state in the Discussion: "While establishing the method we found that injecting liquid into a sperm well causes unwelcome injection artifacts, probably due to dilution of the buffer in the well and/or displacing sperm from the well bottom. The first injection causes the largest injection artefact so we included a mock injection with sperm buffer into all wells at the beginning of the protocol."
This reinforces the perception that the authors do not know how many sperm they have (and on which measurements are made) because attachment is loose and sperm are detaching from the bottom of the well.

See comment above.

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The manuscript has been modified and the updated manuscript, **60815_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

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We checked the manuscript again for spelling and grammar errors.

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We apologize for not removing the full term the first time and replaced Seahorse XFe96 Analyzer with extracellular flux analyzer. We were wondering if it is in accordance with Jove's policies to keep seahorse as one of the keywords. We think this is crucial for a potential reader to find the manuscript. Please let us know if we should remove the term from the keywords as well.

3. Please mention how proper anesthetization is confirmed.

We included this information into the protocol.

4. Please do not highlight any steps describing euthanasia or anesthesia.

We amended the protocol accordingly.

Supplemental File 1

Supplemental File 2

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Supplemental file 2.pzfx

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Author(s):	Melanie Balbach, Jochen Buck, Lonny R. Levin

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