Influence of pulsed electric fields and mitochondria-cytoskeleton interactions on cell respiration

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ABSTRACT

Pulsed electric fields with microsecond pulse width (μsPEF) are used clinically, namely, IRE/Nanoknife for soft tissue tumor ablation. The μsPEF pulse parameters used in IRE (0.5-1 kV/cm, 80-100 pulses, ~100 μs each, 1 Hz frequency) may cause an internal field to develop within the cell due to disruption of the outer cell membrane and subsequent penetration of the electric field. An internal field may disrupt voltage sensitive mitochondria, although the research literature has been relatively unclear regarding whether or not such disruptions occur with μsPEFs. The present investigation reports the influence of clinically used μsPEF parameters on mitochondrial respiration in live cells. Using a high throughput Agilent Seahorse machine, it was observed that μsPEF exposure comprising of 80 pulses with amplitudes 600 or 700 V/cm did not alter mitochondrial respiration in 4T1 cells measured after an overnight post-exposure recovery. To record alterations in mitochondrial function immediately after μsPEF exposure, high resolution respirometry was used to measure electron transport chain state via responses to glutamate-malate and ADP, and mitochondrial membrane potential via response to FCCP. In addition to measuring immediate mitochondrial responses to μsPEF exposure, measurements were also made on cells permeabilized using digitonin, and those with compromised cytoskeleton due to actin depolymerization via treatment with the drug Latrunculin B. The former treatment was used as a control to tease out the effects of plasma membrane permeabilization, while the latter was used to investigate indirect effects on the mitochondria that may occur if μsPEFs impact the cytoskeleton on which the mitochondria are anchored. Based on the results it was concluded that, within the pulse parameters tested, μsPEFs alone do not hinder mitochondrial physiology, but can be used to impact the mitochondria upon compromising the actin. Mitochondrial susceptibility to μsPEF after actin depolymerization provides a novel avenue for cancer therapeutics.

1 INTRODUCTION

The mitochondrial network generates metabolites critical for the biosynthetic and catabolic processes inside the cell. Substrates from food are broken down and ultimately channeled to the electron transport chain (ETC), in which a series of reduction/oxidation reactions along the inner mitochondrial membrane are coupled to proton pumping. The resulting proton gradient creates and sustains mitochondrial membrane potential critical for bioenergetic homeostasis. In mammalian cells, molecular oxygen is the terminal electron acceptor in the ETC. Accordingly, measurement of mitochondrial oxygen consumption provides a reliable measurement of bioenergetic status. Beyond its bioenergetic and biosynthetic functionalities, the mitochondrion is now understood to also act as a signaling organelle. For example, an ETC protein cytochrome C is released by the mitochondrion to initiate cell death. Furthermore, the mitochondrion contains its own DNA and the signaling between the mitochondrion and the nucleus is now thought to regulate many cellular processes including those involved in cancer progression. The interested reader is directed to the review in Chandel (2014). Due to the role of the mitochondrion in cellular processes, particularly in cell death, there is an increasing interest in understanding how applied exogenous transient pulsed electric fields (PEFs) that are used in clinical tumor ablation interact with the mitochondrial network.

A representative clinically used PEF pulse train consists 80 to 100 square wave pulses each of width 100 μs delivered via electrodes into the tissue at a frequency of 1 Hz. Note that the clinical modality using PEFs is known as irreversible electroporation (IRE) or Nanoknife (Jourabchi et al., 2014; Martin et al., 2017; Scheffer et al., 2015, 2016; Scheltema et al., 2016; Trimmer et al., 2015). The delivery of energy via PEFs disrupts the plasma membrane, causing loss of cell homeostasis, which can lead to cell death if the cell does not recover from the perturbation. To target organelles such as the mitochondrion, investigators have suggested leveraging the electrical property of the outer cell bilayer membrane. The permittivity of biological lipid cell membranes decreases as the frequency of an exogenous time-oscillating electric field is increased. Similarly, the conductivity of these membranes increases with an increase in frequency of the exogenous oscillating electric field. Thus, when a cell is exposed to an exogenous square wave electric field, the harmonics (or frequency components) dictate the electrical property of the membrane. It has been theoretically and experimentally argued (e.g., Schoenbach *et al.* (1997) and Ivey *et al.*, (2015)) that reducing the pulse width of the square wave electric field to the order of a sub-microsecond or nanosecond, and thereby increasing the number of high frequency harmonic components, can make the outer lipid membrane conductive to the electric field and allow most of the exogenous field to reach the cytoplasmic compartment. An excellent summary of investigations using exogenous fields with nanosecond pulse widths is given in Napotnik *et al.* (2016). As indicated in the research literature, such an approach of increasing higher frequency components of an exogenous square wave electric field has been proposed as a way of targeting the mitochondria with limited impact on the outer cell lipid membrane.

However, contradictory findings have been reported in terms of the impact of nanosecond PEF (nsPEF) on cytochrome C release by mitochondria with some findings indicating no release and others release. Clearly, it would be of benefit in cancer therapeutics for the latter and not the former to be true. For example, an investigation from 2003 (Beebe *et al.*, 2003) reported exposure of human Jurkat and HL-60 cells to a nsPEF train with varying pulse widths (10-300 ns per pulse) and field strengths (36-150 kV/cm). Note that across all their treatment conditions, an energy density of 1.7 J/cc was delivered. Immediately after exposure, cell fractionation was performed to obtain cytosolic proteins. Immunoblot measurement of cytochrome C revealed an increase in the

protein levels with increasing pulse width. The increased level of cytochrome C in the cytosolic compartment was associated directly with the impact of the nsPEF on the mitochondria. A more recent study (Estlack et al., 2014b) exposed human T-lymphocytes Jurkat cells and monocytes U937 cells to a pulse train of a 100 pulses each with a 10 ns width and 1 Hz frequency. Two field strengths were used: 50 kV/cm and 150 kV/cm. The expressions of caspase 9 and BAX involved in mitochondria mediated cell death was not detected in either cell line upon exposure to the nsPEF. Immunoblot measurements also confirmed no release of cytochrome C in either case.

Contradictory findings have also been made with regard to the impact of nsPEF on mitochondrial membrane potential, which could indicate whether or not the exogenous field permeabilizes the mitochondrial membrane and, thus, influences the proton gradient across it critical for ATP production. For example in Napotnik *et al*. (2012), mitochondrial membrane potential fluorescent dyes rhodamine R123 and tetramethyl rhodamine ethyl ester (TMRE) were used to stain lymphocyte Jurkat cells prior to nsPEF delivery. After the staining and washing steps, cells were exposed to 5 or more pulses each with widths of 4 ns delivered at a frequency of 1 kHz with a field magnitude of 100 kV/cm. Fluorescent signals were captured using a microscope, and raw units were compared before and after exposure to the nsPEF. The investigators reported a reduction by 76% and 78% in the raw units of R123 and TMRE, respectively, upon nsPEF exposure. Moreover, there was an induced change of 61% and 12% in the raw units of R123 and TMRE, respectively, in cells treated with protonophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) as a positive control, which disrupts the mitochondrial membrane potential. Another study using human liver cancer SMMC-7221 cells compared mitochondrial membrane potentials resulting from different microsecond and nanosecond pulse treatments (Mi *et al*., 2009). Similar to the previously mentioned study, cells were stained with the R123 dye and washed before exposure to the PEF. The pulse train for the microsecond PEF consisted of an exposure of 8.5 minutes to 200 V/cm fields and pulse widths of 1.3 μs delivered at a frequency of 50 Hz. The nanosecond pulses had a similar frequency and exposure time with a higher field magnitude (600 V/cm) and a pulse width of 100 ns. Note that this study used an exponentially decaying pulse as opposed to the square pulses used in the other mentioned investigation. As in the previous study, they also report a lowering of the raw fluorescent units in cells treated with either pulse train, although the reduction was steeper in the nanosecond pulse treated cells. This was indicative of the mitochondrial membrane potential being perturbed by both the microsecond and nanosecond pulses.

While the two studies described in the previous paragraph on intact cells provide evidence of the PEF (or more specifically the nsPEF) impacting the mitochondrial membrane potential, they seem to contradict findings on isolated mitochondria by Estlack *et al.* (2014a). In this study, Jurkat and U937 cells were used to isolate mitochondria, which were then exposed to 50 kV/cm and 10-100 pulses each with a 10 ns width delivered at a frequency of 1 Hz. The oxygen consumption was measured using an Agilent Seahorse machine an hour after exposure. The study reported no alterations in mitochondrial respiration with nsPEF exposure, indicative of no effect of the nsPEF train used on mitochondrial membrane potential. This contradiction between findings on intact cells and isolated mitochondria leads to the question of whether or not the differences are due to the complex interactions, both structurally and functionally, that the mitochondria inside the intact cells have with other cell structures such as the cell cytoskeleton on which each mitochondrion is anchored. A possibility, thus, exists that the impact of the nsPEF on the mitochondria is an indirect effect via alterations in some other cellular component. As mentioned earlier, it is hypothesized that the higher frequency harmonics of nsPEFs have limited impact on the outer cell membrane.

It, however, cannot be completely ruled out that nsPEFs do indeed change the intracellular ionic concentrations brought about by cell membrane permeabilization. Such alterations in intracellular environment could also effect mitochondrial respirations.

In contrast, it has been argued that microsecond pulse parameters used in clinical settings do impact mitochondrial membrane potential due to outer cell membrane permeabilization. For example, theoretical predictions by Esser *et al*. (2010) suggest that upon membrane permeabilization, voltage sensitive organelles such as the mitochondria are exposed to the exogenous PEF. However, the question arises as to what μsPEF amplitudes impact the mitochondrial membrane potential or physiology? A study by Reynaud *et al.* (1989) on isolated rat liver mitochondria suggests that the PEF pulse amplitude for microsecond pulse trains would have to be extremely high to alter mitochondrial physiology. Briefly, isolated rat liver mitochondria were exposed to 5 PEF pulses each with a 100 μs pulse width and a frequency of 0.2 Hz. Increased mitochondrial respiration was indirectly indicated by an increased basal oxygen consumption rate for field strengths up to 2 kV/cm, measured immediately after exposure using Clark-type electrodes. Mitochondrial damage indicated by fusion of the mitochondria was only seen beyond 1 kV/cm. However exposure of live cell mitochondria to such high fields may not be practical, as we have previously shown in Goswami *et al.* (2017), a field strength above 700 V/cm with clinically used pulse parameters (width: 100 μs; frequency: 1 Hz; pulses: 80-100) results in very limited cell viability.

Nevertheless, these studies highlight that the influence of nanosecond and microsecond PEFs on mitochondria is still not completely understood. Specifically, a mechanistic understanding needs to be developed on how these PEFs, specifically those used in a clinical setting, impact cell structure and cell signaling. To address this gap in our understanding, the following sections present the results of an investigation of mitochondrial responses in murine triple negative breast cancer 4T1 cells exposed to μsPEFs using clinical parameters previously shown to influence cell signaling (Goswami *et al*., 2017). The goal is to determine whether or not mitochondrial perturbations recorded after μsPEF exposure are unique to an electric field effect or whether similar perturbations can be brought about by non-electrical means. Respecting the structural and functional complexity that exists in a cell, an investigation is also made of whether or not a disruption of the actin cytoskeleton triggers a higher susceptibility of the mitochondria to μsPEF perturbation.

2 MATERIALS & METHODS

2.1 Cell line and culture conditions: The triple negative breast cancer murine cell line 4T1 was purchased from American Type Culture Collection (ATCC, Catalog number: CRL-2539). The 4T1 cells were maintained in an RPMI-1640 culture medium supplemented with 10% (by volume) fetal bovine serum (FBS) and 1% (by volume) penicillin streptomycin (PS). Cells were sustained in humidified incubators at 37 °C and 5% CO_2 and were sub-cultured at approximately 80% confluence, while 0.25% Trypsin-EDTA solution was used for detachment. All experiments were performed within the first ten sub-cultures.

2.2 Pulsed electric field exposure protocol: The protocol was previously reported by Goswami *et al.* (2017). Briefly, following the harvest, the cell pellet was washed with phosphate buffered saline without calcium and magnesium ($PBS^{-/-}$; Santa Cruz Biotechnology) and re-suspended in a basal growth media with neither serum nor PS. Approximately 6-8 million cells/cuvette were transferred to 4 mm electroporation cuvettes (Mirus Bio LLC) in a volume of 600 mL. Each cuvette was placed in a holder through which the cells were exposed to electric field. The electric voltage required for the μsPEF exposure was generated using an electroporation unit (Harvard Apparatus, USA). Short pulses of 100 μs were delivered at a frequency of 1 Hertz. The number of pulses was kept constant at 80. The electric field strength reported in this article was calculated by taking the ratio of electric voltage applied to the distance between the electrodes in the cuvette (4 mm). Two different field strengths were investigated: 600 V/cm and 700 V/cm. The viabilities of the 4T1 cells exposed to these field strengths were reported by our earlier study to be 80% (600 V/cm) and 40% (700 V/cm) when evaluated after overnight incubation post-treatment (Goswami *et al*., 2017). For the untreated controls, cells were transferred to cuvettes and kept in the cuvette for the same duration as the treatment groups without application of an electric field.

2.3 Mitochondrial respiration measurements in an Agilent Seahorse XF machine: After exposure to the PEFs, cells (including the untreated controls) were allowed to recover for approximately thirty minutes in ice while total cell count was made using a hemocytometer for each sample. Approximately 40,000 cells were seeded in each well of an Agilent Seahorse (Santa Clara, California, USA) XF24 tissue culture plate and allowed to incubate overnight in a cell culture incubator at 37 ℃ and 5% CO_2. After overnight incubation, samples were washed with assay buffer (Seahorse XF Base Medium, 10mM pyruvate, 10mM glucose, and 2mM glutamax) after which mitochondrial respiration via oxygen consumption rate (OCR) was measured in the XF24 plate reader following our established protocol (Dai et al., 2016). Injections of 2 μM ATPase inhibitor Oligomycin, 0.5 μM protonophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and 1 μM electron transport chain complex III inhibitor Antimycin-A were made to measure mitochondrial response. All concentrations reported are the final concentrations in each well.

2.4 Mitochondrial respiration measurements via Oroboros O2k respirometry: Oxygen consumption was determined using our established protocols (Alleman et al., 2016). Immediately after exposure to the PEFs, cells were centrifuged at 200 r.c.f. for 5 minutes. The cell pellet obtained was re-suspended in an assay buffer (Buffer Z: 105mM K-MES, 30mM KCl, 10mM KH_2PO_4, 5mM $MgCl_2$-$6H_2O$, 0.5mg/mL BSA, 20mM Creatine, 1mM EGTA, pH 7.1 in dH_2O). Two milliliters of the cell suspension containing approximately 6 million cells was then transferred to the Oroboros O2k respiration chamber. A small volume from the sample was used to determine cell number using a hemocytometer before transferring to the respiration chamber. The O2k respiration chamber is equipped with calibrated oxygen measuring polarographic sensors. The chamber was maintained at 37 °C, and the oxygen signals were allowed to stabilize before closing the chamber to the outside air. After closing, the OCR was measured following no injections (basal), 2 mM of malate and 10 mM glutamate (G/M), 5 mM of adenosine diphosphate (ADP), and two injections of FCCP with a final chamber concentration of 0.3 μM. All concentrations reported are the final concentrations in each well. For control treatments in which the cells were permeabilized using the chemical digitonin, OCR signals were allowed to stabilize after closing the chamber following which digitonin (final concentration: 10 μg/mL) was injected. The basal rate was measured once the signals stabilized after digitonin injection. Measurements of the OCR responses to G/M, ADP, and FCCP were as described above.

2.5 Quantification of cell permeability using cell permeant Calcein: Supernatant from cell culture flasks (growth area: 75 cm^2) was removed, and cells were washed with $PBS^{-/-}$. Following the wash, growth media containing 5 μM cell permeant Calcein AM (ThermoFisher Scientific, USA) was added to the flask (3 mL/75 cm^2). After a thirty-minute incubation in a cell culture incubator, cells were harvested following a standard Trypsin-EDTA harvesting procedure. Final samples were placed in a 0.5 mL basal media, which was followed by treatment and immediate centrifugation at 200 r.c.f. for 5 minutes. Samples were then placed on ice, and the supernatant (100 μL) was collected at 10, 15, and 20 minutes. Fluorescence of Calcein was detected in the supernatant using a spectrophotometer (BioRad, USA). The raw fluorescent unit (RFU) of controls was monitored such that only those time-points at which the RFU of the control did not change considerably were considered for measurements. The permeability was expressed as the fold change of the RFU compared to the controls.

2.6 Actin depolymerization using Latrunculin B: Supernatant from cell culture flasks (growth area: 75 cm^2) was removed, and cells were washed with $PBS^{-/-}$. Following the wash, growth media containing 1 μg/mL Latrunculin B (Sigma Aldrich, USA) was added to the flask. After a ten-minute incubation in a cell culture incubator, the cells were checked under a microscope to verify morphological changes. The supernatant was discarded, and cells were washed again with $PBS^{-/-}$. Next, cells were harvested following a standard Trypsin-EDTA harvesting procedure.

2.7 Statistical analyses: The software JMP® (version 12, SAS Institute Inc., Cary, NC) was used for statistical analyses. Samples were compared using a one-way analysis of variance (ANOVA) followed by a post hoc Tukey HSD to find means that were significantly different from each other.

3 RESULTS

The effects of clinically-relevant μsPEFs on mitochondrial respiration was evaluated via high-resolution respirometry. Briefly, cells in suspension were exposed to PEFs after which cells were centrifuged and suspended in an assay buffer. The cell suspension volume of two milliliters was transferred to the oxygraph chamber after which mitochondrial respiration response to the substrates glutamate-malate, adenosine diphosphate, and protonophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was measured. A comparison of mitochondrial responses between PEF and chemically induced (via Digitonin) permeabilizations of the cell membrane was made. In addition, this investigation also probed into the significance of a nexus between the actin cytoskeleton and the mitochondria.

3.1 PEFs alone do not influence live cell mitochondrial membrane potential: Cell suspensions were exposed to electric field strengths (= applied voltage/distance between electrodes) of 600 V/cm, and 700 V/cm. The pulse duration was kept constant at 100 μs (see **Figure S1** in the supplementary figure section), consistent with clinically used values (Martin II *et al.*, 2012; Thomson *et al.*, 2011). The frequency of the electric fields was 1 Hz and the number of pulses 80. These PEF parameters were not only chosen due to their clinical relevance but also due to our previous observation of these field parameters influencing cell signaling in human and murine triple negative breast cancer cells (Goswami *et al.*, 2017).

A preliminary analysis of the mitochondrial respiration of cells exposed to these PEFs was made after allowing overnight incubation. Briefly, cells were exposed to PEFs and then seeded in tissue culture Agilent XF Seahorse well plates. After overnight incubation, mitochondrial activity was measured via oxygen consumption rates of cells in response to ATPase synthase inhibitor oligomycin, uncoupling protonophore FCCP, and electron transport chain complex III inhibitor

Antimycin-A (**Figure 1A & 1B**). Two mitochondrial performance indices were calculated: respiratory reserve capacity and ATP production capacity. The respiratory reserve capacity is the difference between the OCRs as a response to uncoupling via FCCP and the basal rate. The protonophore FCCP collapses the mitochondrial membrane potential and is indicative of the maximum respiration the mitochondria can achieve. Respiratory reserve is a representative measure of the cell's ability to respond to increased metabolic demands. The OCR measured after inhibition of ATPase activity provides the fraction of basal rate involved in ATP production. Thus, the difference between the OCRs measured at the basal condition and after oligomycin injection provides a measure of the ATP production rate capacity of the cells.

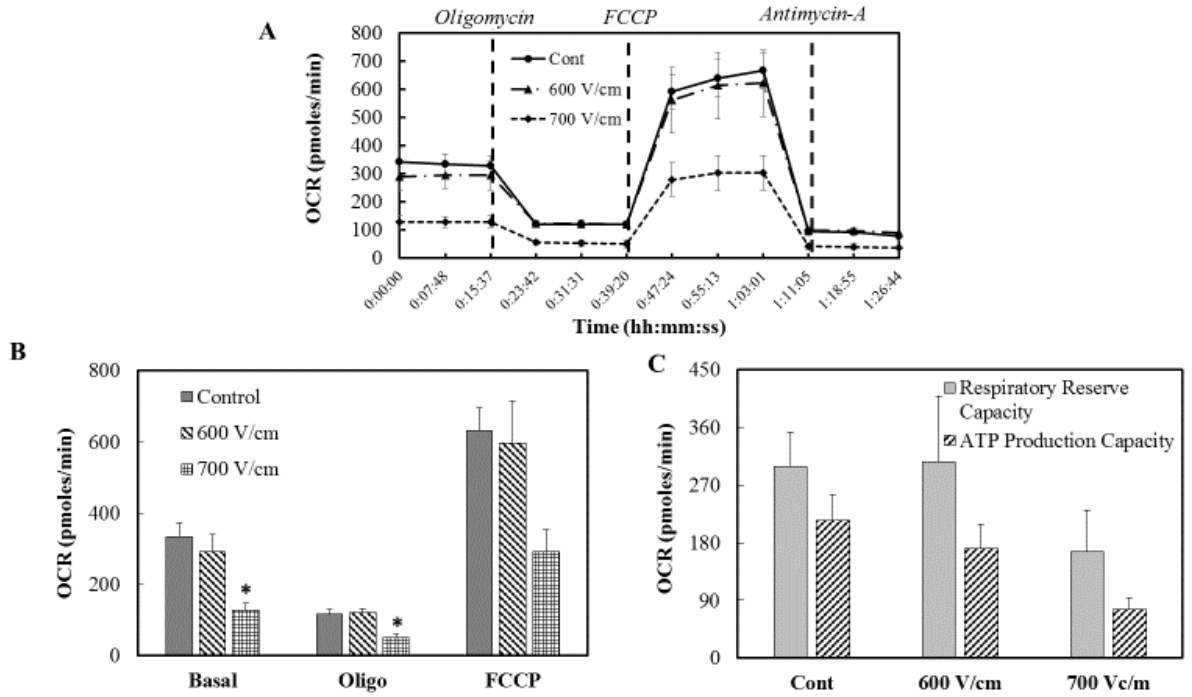

Figure 1: The OCR of live cell mitochondria after overnight incubation post PEF exposure (**A**). The OCR measurements expressed in pmoles/min are summarized in **(B)**, and these measurements were used to calculate the respiratory reserve and ATP production capacities (**C**). One-way ANOVA analysis followed by a Tukey HSD post-test was performed to test for statistical significance. Error bars represent the standard error of the mean, and the number of sets per condition n = 3.

The XF metabolic data (**Figure 1C**) showed no difference in the respiratory reserve and ATP production capacities between the untreated controls and the cells exposed to 600 V/cm. There was a slight (albeit statistically insignificant) decrease in respiratory reserve capacity of cells exposed to 700 V/cm. Moreover, the ATP production capacity of cells exposed to 700 V/cm was lower than the untreated control, though statistically insignificant. Note that our previous measurements of cellular viabilities after overnight incubation (Goswami *et al.*, 2017) indicate that the live fraction of total cells, which survive exposure to PEFs, was determined to be approximately 80% and 40%, for 600 V/cm and 700 V/cm, respectively. This reduction in cell viability is consistent with the reduced absolute respiration rates in cells exposed to 700 V/cm seen in Figures 1B & 1C.

The XF metabolic data provided no conclusive evidence as to whether or not mitochondrial physiology is impacted by PEFs, since it could be argued that the differences in respiration can be

attributed to the fewer live cells in the 700 V/cm group versus the untreated controls. We next proposed that the impact of PEFs on mitochondrial respiration and physiology may only be limited to shorter temporal scales, i.e., immediately after exposure. To test this hypothesis, experiments using high-resolution respirometry were performed on cells exposed to PEFs. Mitochondrial responses to glutamate and malate (G/M), adenosine diphosphate (ADP), and protonophore FCCP were measured via the OCR normalized to live cells (pmoles/s-million units; **Figure 2A**). The glutamate-malate substrate partakes in the complex I activity of the electron transport chain (ETC) in the mitochondria whereby an electron is transferred forward through complexes III-IV. The electron flow is used in a series of reduction steps to convert molecular oxygen to water and establish a proton gradient across the mitochondrial membrane. The mitochondrial membrane potential is largely dependent on this proton gradient, and the proton gradient provides the free energy for the ATPase 'turbines' (Complex V) to phosphorylate ADP to the energy currency adenosine triphosphate (ATP). The responses to G/M and ADP, thus, provide a performance index of the ETC whereas the disruption of the proton gradient via FCCP collapses mitochondrial membrane potential.

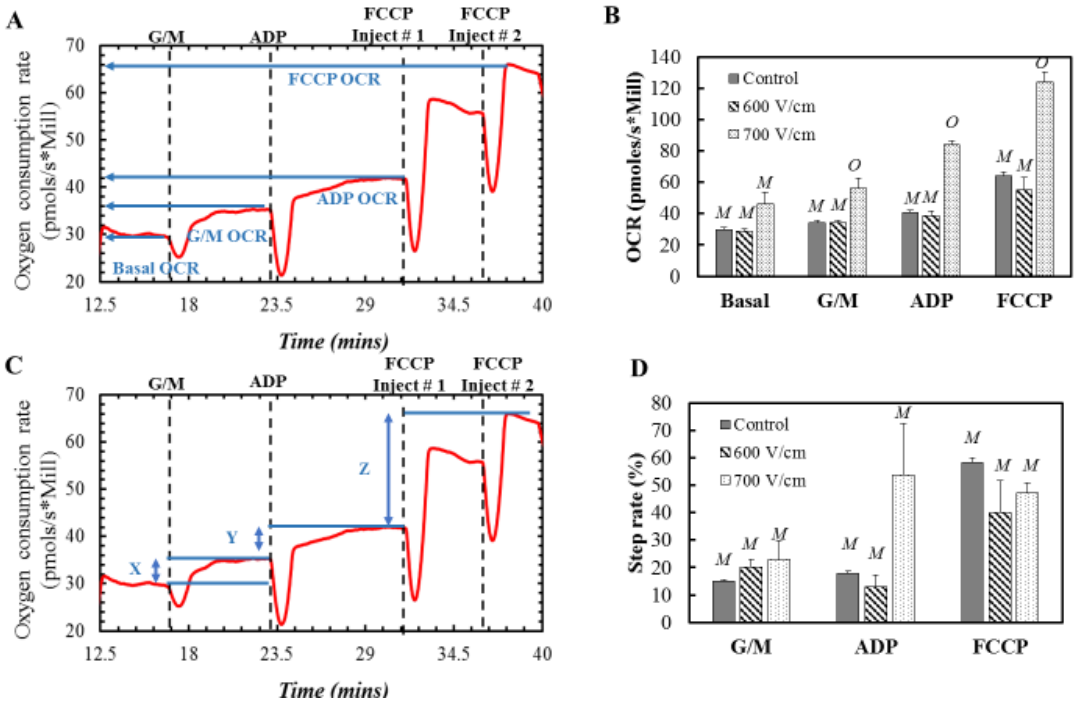

Figure 2: Representative oxygen consumption traces are shown in **Figures A** and **C** as references for **B** and **D**, respectively. The mitochondrial response to glutamate-malate (G/M), adenosine diphosphate (ADP), and FCCP was measured via oxygen consumption rates per million cells as shown in **B**. Another method of analysis is provided in terms of step heights of the metabolic profile in **C**. Error bars represent the standard error of the mean, and the number of sets per condition n = 3. Statistically non-distinct conditions were grouped by letter (e.g. M versus O) for each substrate response, to illustrate significant differences as determined by a one-way ANOVA followed by a Tukey HSD-post test. Note that the dips seen in the respirometry measurements (A & C) are due to disturbances created by the injections.

A representative respirometry plot is shown in **Figure 2A**. The initial equilibrated signal of the cell OCR was recorded as the basal rate. Respiration rates of cells were allowed to stabilize after

each injection of substrate/protonophore before making further injections. Two serial injections of FCCP were made and the highest response was recorded. Normalization of the OCR with live cells was performed by counting the approximate number of total cells via a hemocytometer and assuming the live fraction of the total count (Control: 100%; 600 V/cm: 80%; 700 V/cm: 40%) based on independent viability data we reported with the same cell and pulse conditions (Goswami *et al*., 2017). Thus, the OCR was normalized and expressed in units of pmoles/s-million cells. Based on the experiments, it was observed that the untreated controls and the cells exposed to 600 V/cm did not have significant differences in mitochondrial responses to G/M, ADP, and FCCP (**Figure 2B**). On the other hand, the cells exposed to 700 V/cm had significant differences with both the controls and the 600 V/cm group in all mitochondrial responses. The increased respiration rates after electric field exposure has been previously reported in isolated rat liver mitochondria (Reynaud *et al*., 1989).

A source of error while reporting the normalized OCR is the variability in the total and live cell count. Thus, a normalization technique of the respirometry data independent of the live cell number was deemed useful in countering this variability. Moreover, the OCR after injection of a substrate/protonophore is dependent on the previous OCR step increments. To address these issues, a mathematical expression independent of the number of cells was used to isolate the individual step increments after each substrate injection. This step rate is defined as

$$Step\ rate_i = \frac{OCR_i - OCR_{i-1}}{OCR_{i-1}} \times 100 \qquad \textbf{Eqn. 1}$$

In this expression, the subscript 'i' denotes a substrate (for e.g., ADP) and 'i-1' denotes the substrate used prior to 'i' (for e.g., i-1 for ADP is G/M). Thus, graphically the G/M step rate is the height *X* (**Figure 2C**) divided by the basal OCR (**Figure 2A**) for the sample. Similarly, the ADP step rate is the height *Y* divided by the G/M OCR, and the FCCP step rate is the height Z divided by the ADP OCR. These fractions are expressed as percentages in **Figure 2D**. Moreover, the ratio in Eqn. 1 makes it independent of the number of live cells/mitochondrial mass in the sample. Based on this performance index, it is observed that the PEFs at 700 V/cm induce higher step increments in the G/M and ADP response although these are not statistically significant from the untreated controls (**Figure 2D**). Note, that individual recorded step-rate data for the ADP response in cells exposed to 700 V/cm was higher (31.9, 38.5, 91.2) than that for the controls (19.5, 17.4, 17.1), but possessed high variability due to which a strict statistical analysis would deem the two groups no different from each other. There are lower step increments in FCCP response in both treatment conditions (600 V/cm and 700 V/cm) but again not statistically significant. A lower step-increment could indicate the depolarization of the mitochondrial membrane. Thus, based on the data and within the range of micro-second PEF pulse parameters used, the mitochondrial membrane potential and physiology are not significantly altered.

3.2 Chemical permeabilization of outer cell membrane leads to altered mitochondrial respiration: Higher step rates for the G/M and ADP injections were recorded for cells exposed to the 700 V/cm PEF **(Figure 2)**. A possibility exists that electric fields develop in the cytoplasmic cellular compartment because of permeabilization of the plasma membrane (Esser *et al*., 2010), which, therefore, perturb the functioning of the voltage sensitive ETC. In addition, previous literature (Hamamoto et al., 1982; Teissie, 1986) has reported increased ATP production in an isolated mitochondrion and bacterium when exposed to electric fields similar to those used in this study. However, an alternative explanation is perhaps that the permeabilization of the plasma membrane enhances trans-membrane substrate transport to the organelles.

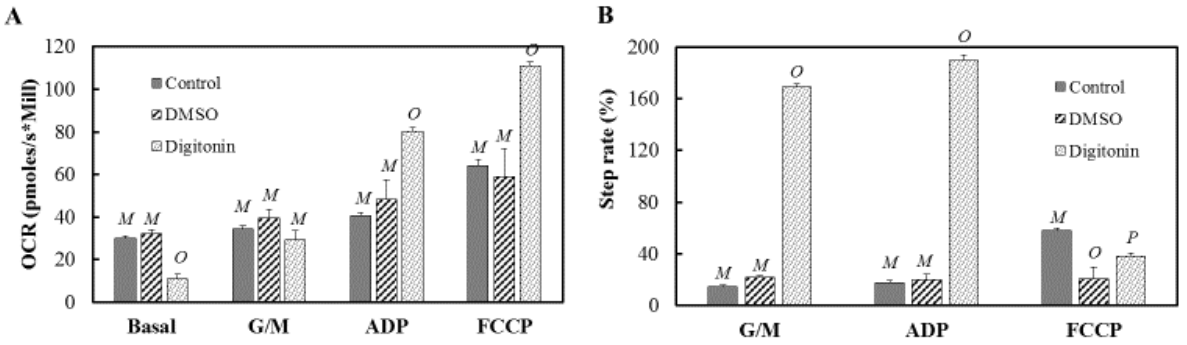

Figure 3: The OCR (**A**) and step rate (**B**) responses to different substrates are shown for cells without any treatment (control) and cells treated with DMSO and digitonin, respectively. Error bars are for the standard error of the mean, and n = 3 for the control and DMSO groups, while n = 4 for the digitonin group. Statistically non-distinct conditions were grouped by letter (e.g., M versus O versus P) for each substrate response, to illustrate significant differences as determined by a one-way ANOVA followed by a Tukey HSD-post test.

To clarify this issue, we quantified the permeability of cells exposed to PEF pulse parameters within the range used in this study. Briefly, cells were loaded with the cell permeant dye Calcein AM. The cell permeant dye is not fluorescent but is cleaved to release fluorescent cell impermeant Calcein upon internalization. After a thirty-minute incubation in a cell culture incubator followed by removal of any residual dye via wash steps with PBS, cells were harvested using Trypsin-EDTA solution and exposed to PEFs as detailed in the methods section. The stained cell sample was then centrifuged, and a pellet obtained (**Figure S2**). A volume of 100 μL was drawn from the supernatant at 10, 15, and 20 minutes, and the fluorescence was read using a spectrophotometer to measure levels of Calcein. Fluorescence units were then normalized to untreated controls for a fold change in raw fluorescence units (RFU). Since the cell impermeant Calcein molecule is expected to leak out of permeabilized cells faster than out of the untreated controls, the fold change provides a semi-quantitative measure of cell permeability. A similar approach to measure cell permeability is reported by Neri *et al.* (2001). Note that although Calcein AM is typically used to measure cell viability, in the present investigation (as is done in Neri *et. al.*) it is only used to measure cell permeability. Based on the method described, the RFU fold change in PEF samples were approximately 2 for 600 V/cm and approximately 2.5 for 700 V/cm (**Figure S2)**.

Next, digitonin detergent (Promega Corporation, USA) was used to permeabilize the cells and measure the mitochondrial response to the same substrates used for the PEF treatment group. Briefly, cells were placed in the oxygraph chamber and basal rates allowed to equilibrate. After equilibration, digitonin was injected to a final concentration of 10 μg/mL. Again, the signal was allowed to equilibrate after which G/M, ADP, and FCCP were injected as in the case with the PEF treatment groups. Since the digitonin stock is supplied in a dimethyl sulfoxide (DMSO) solvent, an equal volume (1 μL; final concentration 7 mM) of DMSO (Sigma Aldrich, USA) was also injected to measure changes due to the vehicle of the detergent. Note, the permeabilization due to the digitonin dosage was measured to be approximately 2 times higher than the untreated controls using the same approach as described above, i.e., the permeabilization was comparable to that observed for the PEF treated samples during the period of the Calcein release measurements.

Respirometry measurements were then carried out and are summarized in **Figure 3**. The plots illustrate OCR and step rate measurements for untreated cells (control) and cells exposed to DMSO and digitonin. The OCR measurements reveal that the digitonin significantly reduced the basal

rates in cells (**Figure 3A**) when compared to the control and DMSO population. Upon examining the step rates (**Figure 3B**), the chemical permeabilization induces a higher G/M response, which leads to an insignificant difference in the OCRs between the controls and the digitonin treated groups (**Figure 3A**). The digitonin permeabilized cells have higher ADP responses when compared to the other two populations (**Figures 3A** and **3B**). A lower recovery was recorded, as seen in the step rate data, for the chemically permeabilized cells when perturbed with FCCP (**Figure 3B**). Thus, permeabilization via a non-electrical means also leads to increased responses to the substrates and a reduced response with FCCP injection. An important distinction, however, is the opposite directionality of the alteration in basal rates with the electrical and non-electrical permeabilization and the much more pronounced G/M and ADP responses with the digitonin permeabilization when the step-rate data was compared.

3.3 Actin cytoskeleton and the mitochondrial nexus could dictate mitochondrial response to PEFs: The conflicting findings on the effects of PEFs on mitochondrial physiology in experiments with intact cells versus isolated mitochondria leads to the question of whether or not there are indirect secondary effects on the mitochondria due to alterations in other cellular structures. The mitochondria are anchored onto the cell cytoskeleton in specialized cytoplasmic domains that allow them to partake in regulatory and energetic processes. For example, mitochondrial positioning in microdomains inside the cytoplasm allows calcium dependent mechano-sensing in cardiac cells. For a review, the reader is directed to Schönleitner *et al*., (2017). Aberrations in the localization are implicated in disease. Thus, we next explored the possibility of alterations in the mitochondrial membrane potential with PEFs following chemical alteration of the cytoskeleton. Recent reports (Berghöfer *et al*., 2009; Pakhomov *et al*., 2014) have demonstrated that PEFs with high magnitude electric field strengths (19-30 kV/cm; pulse width: order of ns; number of pulses: 1-4) cause structural damage to the cytoskeleton, including depolymerization of the actin structures (Pakhomov *et al*., 2014). We therefore hypothesized that an alteration in mitochondrial function could be achieved with relatively low field strength PEFs (~700V/cm) after pre-treating the cells with an actin depolymerizing agent.

To test this hypothesis, cells were treated with Latrunculin B (LanB) to depolymerize the actin cytoskeleton. The LanB forms a complex with the globular (G)-actin monomer and prevents the polymerization to filamentous (F)-actin (Morton *et al*., 2000). Briefly, cells were treated with 1 µg/mL LanB for 10 minutes. Fixed staining of the cells with the Alexa Fluor Phalloidin conjugate (ThermoFisher Scientific, USA) for F-actin and the nuclear dye DAPI (Sigma Aldrich, USA) demonstrated that the dosage of LanB for 10 minutes was sufficient to compromise the F-actin structures of the 4T1 cells (**Figure 4A**). Following incubation with LanB, cells were washed with $PBS^{-/-}$ to make sure no residual drug remained. Cells were then harvested and exposed to a PEF at 700 V/cm immediately after which respirometry measurements were made as described in the previous sections. Note that an applied field of 700 V/cm does not imply an equivalent electric dosage between those treated with LanB and those that were not. Furthermore, the cell size directly impacts the influence of the electric field on the plasma membrane *(Goswami et al., 2017; Schoenbach et al*., 2007), and, therefore, cell size measurements were made. Both untreated controls and LanB treated cells had a comparable mean diameter. A probability density function of the sizes is provided for reference in **Figure S3**. However, one cannot rule out the impact that LanB may have on other lipid reorganization events which may impact the electric field sensed by the cell across the membrane. Since the LanB stock was prepared in DMSO solvent, respirometry was performed on cells incubated with a volume of the vehicle (final concentration 14 mM) equal to that of the LanB. Note that the OCR data normalized to live cells is not provided for this section

of the study due to challenges in determining live cell fractions immediately after administration in combinatorial treatments involving PEFs. The challenges are discussed in the section that follows. The step rates obtained from the respirometry measurements are shown in **Figure 4B**. The step rate data of untreated cells (control) and vehicle DMSO are provided for reference.

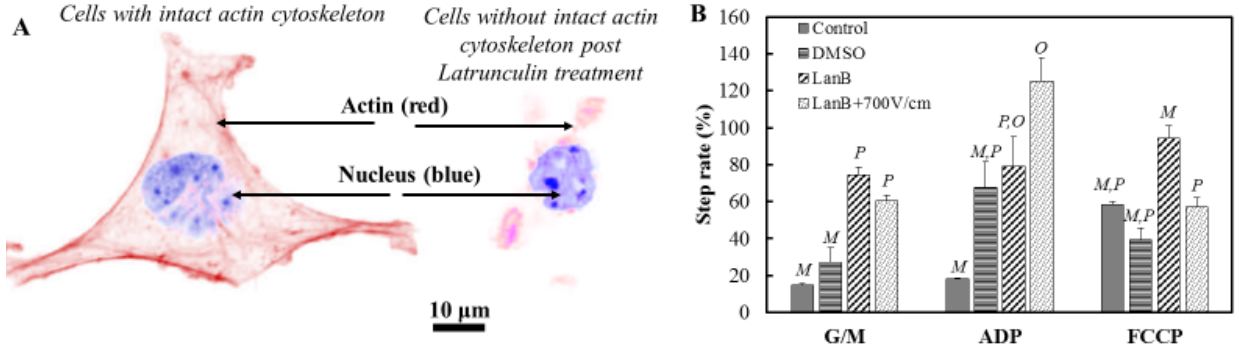

Figure 4: Treatment with Latrunculin B compromises the actin cytoskeleton as shown in the fixed cell staining image (**A**) of the actin (red) and the nucleus (blue). A10 µm scale bar is shown. Shown in **B** is the step rate data for cells with no treatment (control), those treated with the Lan B vehicle DMSO, those treated with Lan B, and those with PEF exposure post the Lan B treatment. The error bars represent the standard error of the mean, and the sample size per set is n =3 for all except the LanB+700V/cm (n=4) treatment. Treatment conditions connected by different letters (M, O, and P) within each substrate response are statistically different as determined by a one-way ANOVA followed by a Tukey HSD-post test.

The step rate data (**Figure 4B**) suggests that treatment with LanB leads to an increased G/M response when compared with untreated controls and cells treated with vehicle DMSO. Upon exposure to a PEF field of 700 V/cm, cells compromised of their actin cytoskeleton via LanB treatment did not significantly differ in their G/M and ADP responses when compared to cells treated with LanB alone. However, actin disruption and PEF exposure reduced the response of cells to FCCP when compared to cells only treated with actin depolymerizing agent LanB. Thus, the step rate data suggests that relatively low field strength PEFs can impact the mitochondrial membrane potential upon depolymerization of the actin as seen by the comparison of the FCCP responses between LanB and LanB+700V/cm groups (**Figure 4B**).

4 DISCUSSION

In this study, murine triple negative breast cancer 4T1 cells were exposed to PEF parameters typically used in a clinical setting. The pulse train used consists of 80 to 100 pulses each with a 100 µs width applied at a frequency of 1 Hz. Note that this clinical modality is also known by the names of irreversible electroporation (IRE) or Nanoknife. The cells were exposed to these µsPEFs and the mitochondrial states measured via either an Agilent Seahorse machine or high resolution respirometry to determine whether or not these types of microsecond pulses significantly alter mitochondrial physiology.

As a first step, this investigation measured the mitochondrial respiration in cells exposed to 80 µsPEF pulses with amplitudes of 600 and 700 V/cm and incubated overnight. The rationale of choosing these field strengths is two fold. First, there is an immediate severe loss of viability of 4T1 cells at field strengths above 700 V/cm. Thus, an understanding about mitochondrial alterations is lost at these higher strengths due to the damage experienced by the entire cell. Second, it has recently been demonstrated that at field strengths of 600 and 700 V/cm the µsPEF pulse train

induces cell signaling alterations in both murine and human breast cancer cells towards a less immunosuppressive inflammatory state as determined by the reduction of thymic stromal lymphopoietin (TSLP) signaling (Goswami *et al.*, 2017). The measurements of mitochondrial respiration after overnight incubation were performed using an Agilent Seahorse machine. Note that it is not necessary for the mitochondrial physiology in surviving cells to remain similar to the controls. For example, Zhang *et al.* (2014) reported mitochondrial hyperpolarization in viable cells 24 hours after treatment with a drug. Given that the cells that escape μsPEFs had altered cell signaling as reported by Goswami *et al*. (2017), the objective of this investigation was to determine if alterations in mitochondrial respirations are also observed in cells that survive μsPEFs when compared to untreated controls. The data (**Figure 1**) gathered in the present investigation suggests that there are no significant alterations in the mitochondrial physiology in the cells that escape or recover from the μsPEF treatment. This finding is in agreement with that reported by Estlack *et al.* (2014a), who used an almost identical Seahorse protocol and found no alterations in mitochondrial respiration in Jurkat and U937 cells exposed to nsPEFs and allowed to recover over a period ranging from an hour up to as long as 24 hours after exposure. Thus, it may be supposed that the effects of these types of nsPEF and μsPEFs on mitochondrial respiration, if any, are short lived (i.e., below one hour).

To test whether or not mitochondrial perturbations exist immediately after μsPEF exposure, the mitochondrial respiration of 4T1 cells was measured in the present study promptly after treatment with the aforementioned field strengths using high resolution respirometry. Cell response to injections of G/M and ADP were made to probe the state of the electron transport chain (ETC). The changes in oxygen consumption rate (OCR) in response to G/M reflect alterations in the complex I activity of the ETC whereas ADP responses provide a measure of the cell's capacity to produce ATP. A final injection of FCCP was made to measure the maximal mitochondrial respiration. When comparing the responses of control and treatment groups to FCCP, a lowered response in one implies a smaller proton gradient across the mitochondrial membrane and, therefore, a lower mitochondrial potential compared to another group. The OCR data are dependent on the mitochondrial mass/number, and, thus, in this investigation, the OCR is normalized to an estimate of the number of live cells based on independent measurements. To circumvent the artifacts that may be introduced due to this cell number and mitochondrial mass dependence, the data is also presented as step rates, whereby an automatic normalization is made within the sample. Such a step-rate approach is very similar in nature to how data using fluorescent dyes to measure mitochondrial membrane potential are reported where the alteration in membrane potential is measured in terms of changes in the fluorescent units before and after a treatment.

4.1 Summary of the respirometry data and a comparison with the literature: In this investigation, the respirometry measurements on cells exposed to μsPEF show increased basal OCR rates 55% higher (albeit statistically not significant) for field strength of 700 V/cm when compared to the untreated controls (**Figure 2B**). Increases in the normalized OCR data were also observed in G/M (63%), ADP (107%), and FCCP (93%) responses for the μsPEF-exposed population when compared to the untreated controls ($p<0.05$). Conversely, while the step-rate response changes measured were not statistically significant, the trends were that while the G/M and ADP responses in the μsPEF treated cells increased when compared to the controls, the FCCP response was 18% lower. To explain these respiration responses, we next considered the enhanced permeabilization of the outer membrane that we independently measured for the 600 and 700 V/cm conditions (**Figure S2**). Based on the existing literature, a number of plausible scenarios may be responsible for these response alterations due to outer cell membrane permeabilization. They are

discussed below.

A first scenario is that the permeability of the cell's outer membrane promotes an unhindered flow of ions and substrates from the extracellular compartment, thereby leading to an increase in respiration due to both G/M and ADP more easily reaching the mitochondria. To recreate this scenario in our experiments, we used a chemical agent digitonin that specifically permeabilizes the outer cell plasma membrane without affecting the nuclear envelope (Griffis *et al*., 2003; Tissera *et al*., 2010). The digitonin permeabilized cells showed decreased basal rates when compared to the untreated controls (**Figure 3A**), which Vercesi *et al*. (1991) claim may be due to a reduction in the adenine nucleotides resulting in a non-phosphorylating state of the mitochondria. Due to the lower basal rates initially, the G/M response of the digitonin-permeabilized cells was lower by 14% for the normalized OCR data when compared to the G/M response of the untreated controls. The analysis of step rate data (**Figure 3B**), however, provides a clearer picture, showing an increased respiration (> 10 fold compared to the controls) in response to the G/M and ADP injections in the digitonin-permeabilized cells. While the G/M and ADP responses can be explained due to an unhindered flow of substrates into the cell, the question, nonetheless, arises as to how the mitochondria may be depolarized, as measured by the FCCP response, due to the permeabilization of the cell's outer membrane? In our study, a significant decrease in FCCP step-rate response was recorded in the digitonin-permeabilized cells when compared to the control, suggesting an impact on the mitochondrial membrane potential. Previous measurements on isolated mitochondria of their electrical properties by Pauly *et al*. (1960) and Schwan (1959) show an almost linear dependence of the internal conductivity of the mitochondria on changes in the external ionic concentrations. Of course, this relationship may not be linear in live-cell mitochondria. Nonetheless, an alteration in the cytoplasmic ionic concentrations due to the permeabilization of the cell's outer membrane and resulting changes in the electrical properties of the mitochondria would be consistent with the FCCP responses we report.

A second scenario is that exogenous electric fields, which gain access into the cell's interior after outer membrane permeabilization, directly affect the mitochondria, contributing to our observed increases in respiration at 700 V/cm. Based on the existing literature, it is thought that an exogenous electric field can either lead to an increase in ATP production by the mitochondria or lead to an alteration in the mitochondrial physiology (i.e., via mitochondrial membrane permeabilization and/or fusion). In experiments conducted by Hamamoto *et al*. (1982), isolated rat liver mitochondria were exposed to PEFs with pulse widths ranging from 100 μs to 10 ms at intervals of 30 s. Hamamoto *et al*. reported an increase in ATP synthesis in the mitochondria upon exposure to the PEFs as measured via esterification of the radioactive inorganic phosphate. Moreover, the production increased with increases in both the pulse number and field strengths (> 400 V/cm). In their work, the authors claim that the reason for the increase in ADP to ATP turnover with electric fields is due to the artificially imposed ion gradients across the mitochondrial membrane, thus influencing the membrane potential. In addition, they noted that disruption of the mitochondrial membrane potential via FCCP injection disabled the influence of the electric field in stimulating ATP production. This observation of reduced ATP production in the compromised mitochondria of Escherichia coli bacteria exposed to μsPEFs has also been reported elsewhere (Teissie, 1986). Therefore, based on this literature, as long as the mitochondrial membrane potential does not decrease due to the presence of the electric field, an increase in ATP turnover is possible.

Yet another scenario is that indirect effects on the mitochondria due to a cell's outer membrane permeabilization may occur via alterations in other cellular structures. The disruption of cell

homeostasis due to permeabilization could lead to cell swelling and as a result stress the cell cytoskeleton. For example, actin disassembly due to outer cell permeabilization and cell-swelling was reported by Pakhomov *et al*. (2014) when CHO-K1 cells were exposed to nsPEFs (600 ns pulse widths, 2 Hz frequency, ~20 kV/cm). Two other investigations (Berghöfer *et al*., 2009; Hohenberger *et al*., 2011) in the literature report actin disassembly in plant cells exposed to nsPEFs. However, these latter reports only mention cell permeabilization and not cell swelling. To explore the direct impact of actin disassembly on mitochondrial respiration, we used the drug LanB to depolymerize the actin cytoskeleton. Once the actin network was compromised, the cells were exposed to μsPEF fields of 700 V/cm. The step-rate data (**Figure 4B**) sheds light on how low-level fields (i.e., low-level when compared to the ~kV/cm fields used by the studies above) can impact the mitochondrial membrane potential once the actin cytoskeleton, on which the mitochondria are anchored, is compromised. It is noted that Thompson *et al.* (2014) demonstrated that treatment with Latrunculin A altered cell membrane rigidity in CHO cells making them more susceptible to nsPEF pulses of 150 kV/cm. However, it is difficult to draw specific conclusions regarding the details of mitochondrial effects from the Thompson *et al.* data. In contrast, the present investigation, a link is provided between actin dynamics and the susceptibility of the mitochondria to μsPEF using detailed mitochondrial respirometry experiments. Although the specific mechanism remains to be explored in future work, a hypothesis may be derived as to why actin depolymerization may impact mitochondria more when exposed to μsPEF based on the existing literature. Actin filament dynamics play an important role in apoptotic pathways (Desouza *et al.*, 2012) such as anoikis. The pro-apoptotic protein Bmf can translocate from the myosin V actin motor complex to the mitochondria in the event of activation of anoikis and neutralize the anti-apoptotic protein Bcl-2 (Puthalakath *et al.*, 2001). It has also been demonstrated that activation of the caspase cascade leads to fragmentation of the actin cytoskeleton into 31 kDa Fractin and 14 kDa tActin. Transfection of tActin into mammalian cells results in morphological alterations (for example, cell rounding) associated with apoptosis, indicating a role for actin fragmentation in downstream caspase signaling pathway (Mashima *et al.*, 1997, 1999). In addition to acting as a substrate for the caspase pathway, actin cytoskeleton dynamics can also initiate caspase signaling via both extrinsic and intrinsic apoptotic pathways. For example, CD95/FasL mediated apoptosis was shown to be dependent on the interaction between the actin associated ezrin protein and FasL (Parlato *et al*., 2000). On the other hand, changes in the actin filament dynamics via point mutations in the yeast actin isoform have been shown to induce the accumulation of reactive oxygen species (ROS) and mitochondrial membrane depolarization involved in intrinsic apoptotic pathways (Gourlay *et al*., 2004). Alteration in the actin filament dynamics brought about by treatment with the drug family Latrunculin and Cytochalasin D have been shown to induce the translocation of pro-apoptotic Bcl-2 proteins to the mitochondria and induce mitochondrial depolarization. In addition, experiments on Arabidopsis root hair treated with LanB conducted by Wang *et al*. (2010) reveal that actin disruption leads to an opening of the mitochondrial permeability transition pore which in turn results in a surge in calcium release by the mitochondria. Calcium imbalance in the cytoplasm can alter mitochondrial physiology. It has also been noted that fragmentation into tActin is thought to induce an apoptotic positive feedback loop via cleavage of the pro-apoptotic protein Bid (Slee *et al*., 2000). The nearly 40% reduction in OCR we observe in response to FCCP injection in the μsPEF-treated cells (LanB + 700 V/cm; **Figure 4B**) when compared to the controls (LanB) would be consistent with mitochondrial membrane potential changes enhanced due to actin disruption. Remarkably, Estlack *et al.* (2014b) demonstrated that nsPEFs can modulate extrinsic-mediated apoptotic pathways via the CD95/Fas receptor, although intrinsic pathways involving

the mitochondria were unaffected. Thus, there remains a largely unexplored area of actin-mitochondria-cell membrane nexus interactions that may dictate PEF sensitivity of cancerous cells providing a promising avenue for future work.

In the context of these three scenarios and keeping in mind that the present investigation observed neither a drastic decrease in FCCP response nor a drastic increase in ADP turnover in cells exposed only to μsPEFs (**Figure 2**), *it may be concluded that mitochondrial membrane potential and physiology are not drastically altered by the μsPEF pulse parameters used here*.

Note that the effect of substrates and inhibitors (i.e., G/M, ADP, FCCP, etc.) depend on the sequence in which they are applied in an experimental study. Therefore, the relative changes in mitochondrial membrane potential reported in this study are relative changes between two treatments and not absolute values. While the effects of the sequence of substrates and inhibitors was not explored here, mechanistic computational models of mitochondrial bioenergetics combined with the results from the present investigation can guide the formulation of future hypotheses. One such class of computational frameworks uses thermodynamic flux-force relationships to model the coupled transport of mass and charge across the mitochondrial membrane and, thus, predict the influence that each flux-force term (for example, the flux of Ca^{2+} across the membrane) can have on the kinetics of mitochondrial membrane potential (Beard, 2005; Bertram *et al*., 2006; Magnus and Keizer, 1997). Integration of such models with experimental studies have teased out many interesting facets of the link between mitochondrial bioenergetics and apoptosis. For example, Huber *et al.* (2011) demonstrated that increased glycolysis by cancerous cells generated ATP that supported flux reversal of the ATP synthase and thereby repolarizing the mitochondrial membrane potential even in the presence of the adverse conditions normally associated with cell death (cytochrome c release). Such repolarizations allow the cancerous cells to escape apoptotic events. This highlights the need to investigate the role of the tumor microenvironment, changes in cellular glycolytic capacity, as well as different phenotypes of cells on mitochondrial response to μsPEFs.

Our studies provide interesting insight into the bioenergetic responses following two different approaches to acutely permeabilize the plasma membrane (using μsPEF and Digitonin). Both methods lead to similar cellular rates of ADP- and FCCP-dependent respiration, with each treatment roughly doubling the OCR rate compared to the respective control (Figures 2B and 3A). These data suggest that the *acute* treatment with detergent or PEF is not leading to drastic alterations in mitochondrial membrane potential or ATP-generating capacity by the respiratory chain. These findings are in contrast to our studies after overnight incubation post-PEF exposure, where 700V/cm tended to decrease (albeit not statistically significantly) mitochondrial energetics. It seems plausible that acute buffering of cellular ATP pools by glycolysis may sustain energetics during the acute stressors (as described in Huber *et al*. (2011)), but that this buffering capacity may have been exhausted after overnight incubation. There are also interesting differences in mitochondrial energetics between acute permeabilization and acute μsPEF, notably the large step changes after the addition of glutamate/malate and ADP (in Digitonin compared to μsPEF). We cannot rule out that paradigm-specific differences in the extent/duration of plasma membrane permeabilization led to a cellular "wash-out" of endogenous co-factors that influenced basal and glutamate/malate-mediated respiration, which may explain the variance observed following the detergent treatment. Future studies to further probe these ideas, perhaps in conjunction with studies using computational models (Beard (2005); Bertram *et al*., (2006); Magnus and Keizer, 1997)), should advance our understanding of the similarities and differences among these models.

However, an important finding in our investigation with implications for the use of PEFs (or IRE/Nanoknife) in cancer therapeutics is that of the relationship between the actin networks and mitochondrial physiology. This is discussed in the following.

4.2 Implications for cancer therapeutics: The clinical site of PEF (or IRE/Nanoknife) delivery in tissue experiences a heterogeneous field leading to both lethal and sub-lethal zones. While lethal zones are defined by complete cell death in the region of high electric field exposure, sub-lethal zones experiencing low-level electric fields persist around the tumor margin where malignant cells may exist. Moreover, the treatment zone is limited in volume by a tradeoff between the high electric field magnitudes required to kill cells (~1000 V/cm) and the magnitude of fields that may be safely delivered clinically without inducing deleterious side effects such as muscle contractions. It would, therefore, be highly advantageous to be able to increase the tumor treatment volume and/or induce changes in cell signaling towards an anti-tumor phenotype within a larger sub-lethal treatment volume (Goswami *et al*., 2017). To do so, chemo or molecular targeted therapies can be leveraged in conjunction with PEF exposure as is done in electrochemotherapy (ECT) (Kunte *et al*., 2017; Plaschke *et al*., 2017) to target sub-lethal zones. For example, exposure of 3D spheroids to both PEFs and calcium has been shown to specifically inhibit the growth of tumorous cells rather than healthy fibroblasts (Frandsen *et al*., 2015). Similar pulse parameters as in the present investigation have been used to make glioblastoma cells more susceptible upon PEF exposure combined with calcium loading (Wasson *et al*., 2017). Ivey *et al*. (2017) recently reported that cancerous cells were specifically killed by PEFs over healthy cells upon induction of cell morphological changes brought about via molecular targeting of the EphA2 receptor on human glioblastoma cells. Our current study suggests that molecular adjuvants targeting actin cytoskeleton could be used in conjunction with PEFs to induce cellular death even with low-strength electric fields by further perturbing the organelles such as the mitochondria. While high-strength electric fields (60-300 kV/cm) have been known to cause damage to the actin cytoskeleton and DNA fragmentation leading to cell death (Stacey *et al*., 2003, 2011), molecular adjuvants such as LanB may enhance the kill zone even at low electric field strengths such as those used in our investigation. However, a mechanistic view must be derived to understand the synergistic effects of actin cytoskeleton disruption and PEFs on mitochondrial respiration and the promotion of cell death.

In this regard, several mechanisms by which LanB and related molecules may induce cell death are relatively well documented. Treatment with LanB, in the range from 0.01 to 10 μM, for 10 minutes has been reported to cause DNA fragmentation leading to programmed cell death in pollen cells when assessed 8 hours after treatment (Thomas *et al*., 2006). In human gastric carcinoma cells, treatment with Latrunculin A (LanA) led to increased caspase-3/7 activity when assessed 24 hours after treatment. Treatment of these cells with concentrations between 1-10 μM led to cell viabilities in the range of 20-60% (Konishi *et al*., 2009). Another study on human breast cells found that treatment with LanA led to cleavage of the poly (ADP-ribose) polymerase protein involved in DNA damage repair (Martin and Leder, 2001). It would, therefore, be reasonable to assume that the combination of LanB to disrupt the actin cytoskeleton and PEF exposure would induce a higher cell death rate than the separate treatments alone.

It is worth noting that there are important remaining open questions for future work. A report by Xiao *et al*. (2011) demonstrated outcomes that at first do not seem clearly reconciled with those that we report here. In their study, Xiao *et al*. exposed human liver cancer cells, pretreated with an actin-depolymerizing drug Cytochalasin B, to nsPEFs (450 ns pulse widths delivered at a frequency of 1 Hz and 8 kV/cm). For their measurements of cell death, Xiao *et al*. used the fact

that cells in the early stages of cell death translocate a protein phosphatidylserine from the inner leaflet of the cell membrane to the outside. This expression of the protein on the cell outer membrane can be detected via Annexin V staining. Surprisingly, Xiao *et al*. report that cells with disrupted actin networks and exposed to PEFs had a reduced percentage of the population expressing this protein when compared to PEFs alone. The reason for this observation is not understood, although it may be noted that Annexin V staining on cells performed immediately after PEF permeabilization may provide false positives since the molecule Annexin V may be able to access the interior of the cell due to cell membrane permeabilization caused by the PEF. The primary reason for cellular death with PEFs is hypothesized to be the loss of homeostasis due to a compromised outer membrane. However, the exact mechanism of cellular death caused by PEFs is still debated (Deipolyi *et al*., 2014; Maček-Lebar and Miklavčič, 2001). The potential for reversible permeabilization of cells, in which cells recover from the loss of homeostasis, due to PEFs also makes it difficult to ascertain live cell populations using simple dyes such as Trypan Blue immediately after PEF treatment. However, we are hopeful that new cancer therapy insights will result from identifying the specific mechanisms regulating cellular responses to combinatorial treatments such as those we investigate here.

5 CONCLUSION

There is a limited understanding of whether or not μsPEFs based on the IRE/Nanoknife clinical treatment modality directly impact the cell mitochondrial physiology of the treatment zone. To address this gap in knowledge, the present investigation provides measurements of the mitochondrial state via high resolution respirometry on 4T1 cells exposed to μsPEF pulse trains that are used in clinical settings. In these measurements, the ETC state of the mitochondria is probed via responses to G/M and ADP. Additionally, changes in the mitochondrial membrane potential are inferred via responses to FCCP. It is observed in the present study that the impact of μsPEFs on mitochondrial respiration is short (< 1 hour) and temporary. Moreover, it is proposed here that these alterations are primarily due to the permeabilization of the cell's outer (plasma) membrane. Comparison between responses of cells exposed to μsPEFs versus the chemical permeabilization agent digitonin indicates that clinically used μsPEFs do not significantly alter mitochondrial physiology within the pulse parameters tested. However, our finding of the mitochondria's susceptibility to sub-lethal μsPEFs when the cell's actin cytoskeleton is compromised via the actin depolymerizing agent LanB could provide both mechanistic insights as well as avenues for increasing the IRE/Nanoknife treatment volume in a clinical setting.

AUTHOR CONTRIBUTIONS

I.G.: study design, cell culture, respirometry experiments, data analysis and interpretation, and writing of manuscript; J.B.P: design of O2k experiments, and writing of manuscript; M.A.A: design of XF Seahorse experiments, and writing of manuscript; D.A.B.: data analysis and interpretation, and writing of manuscript; M.R.v.S.: conception of project plan and study design, data analysis and interpretation, and writing of manuscript; S.S.V.: conception of project plan and study design, data analysis and interpretation, and writing of manuscript.

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SUPPLEMENTARY FIGURES:

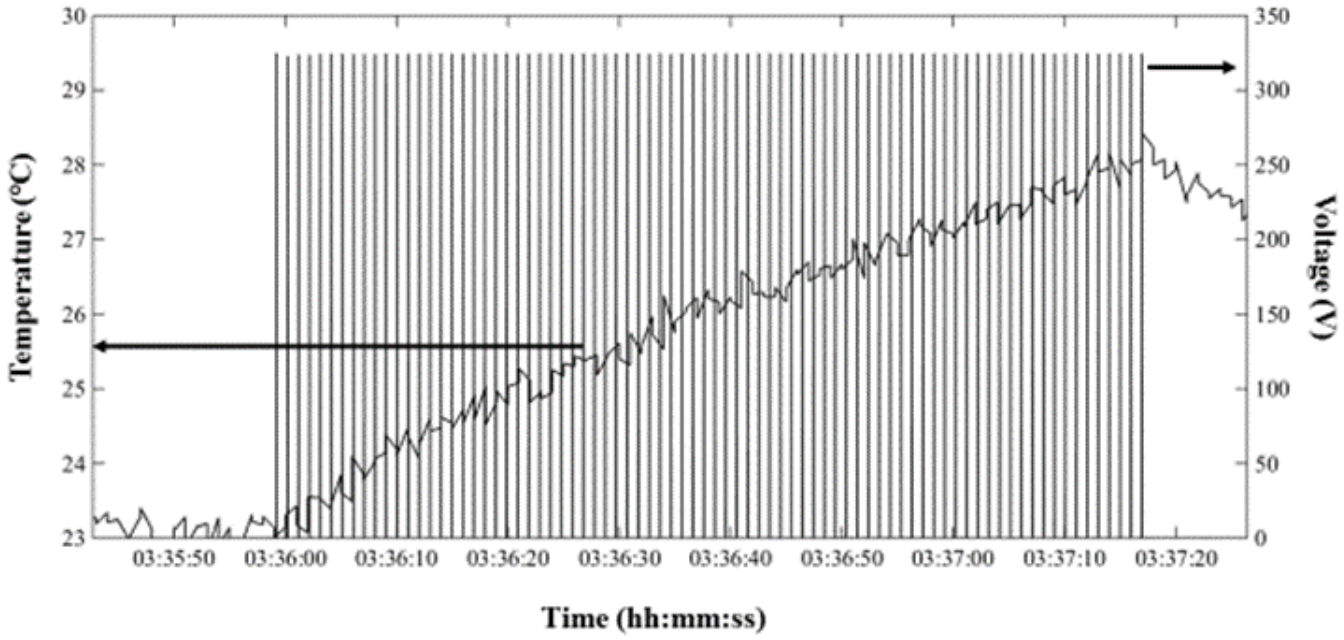

Figure S1: A representative real-time signal captured during treatment showing the profiles of voltage and temperature during pulsed electric field exposure. The voltage signal comprised of 80 pulses (represented by the thin lines) each 100 micro-second wide, and a frequency of 1 Hertz.

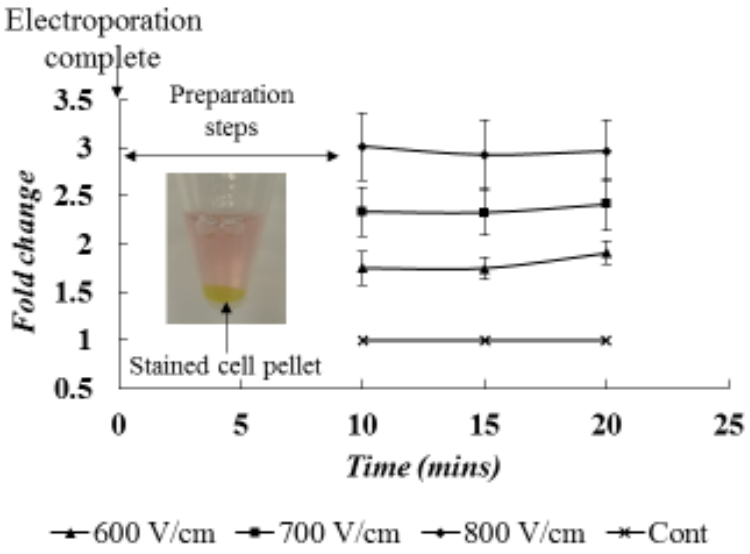

Figure S2: The quantification of cell permeability using Calcein AM assay is shown for cells exposed to PEFs with different field amplitudes.

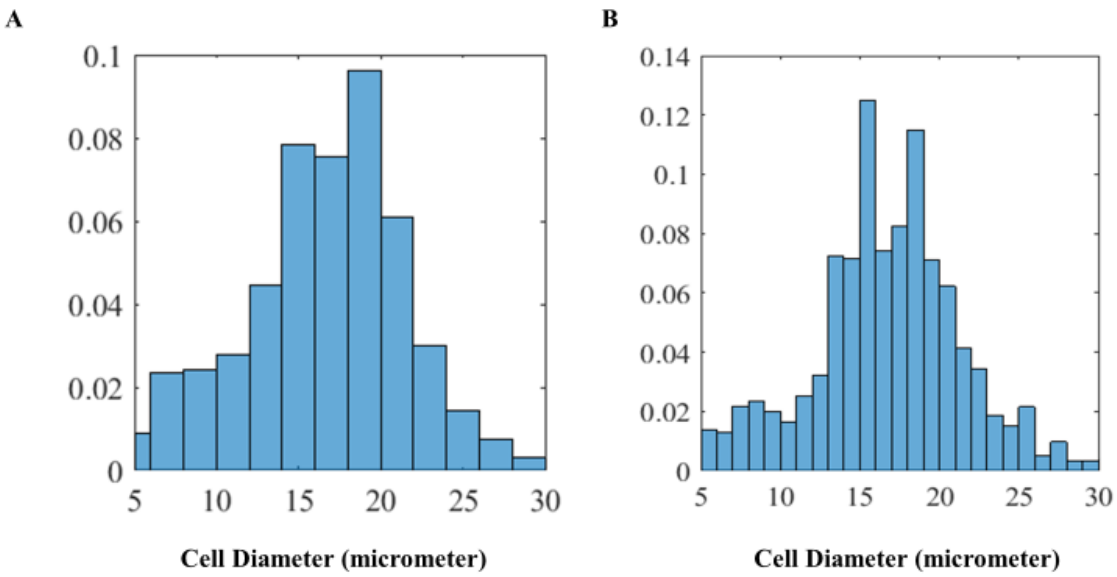

Figure S3: Probability density function of cell diameter in untreated cells (A) and cells treated with Latrunculin B (B) upon harvesting. The mean size of the cells in suspension between the two groups remained comparably similar.