Role of metabolic pathways and sensors in regulation of dendritic cell-driven T cell responses

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Analysis of TLR-Induced Metabolic Changes in Dendritic Cells Using the Seahorse XF^{e}96 Extracellular Flux Analyzer

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Abstract

Engagement of Toll-like receptors (TLRs) on dendritic cells (DCs) triggers the expression of a large set of genes involved in DC activation and maturation, which allow them to act efficiently as antigen-presenting cells. Recently, it has become clear that TLR signalling in DCs also results in dramatic metabolic changes that are integral to their changed biology. Here, we describe a detailed protocol on how DC metabolism can be studied after TLR stimulation using the 96-well format Extracellular Flux (XF^e96) Analyzer from Seahorse Bioscience, a machine that allows one to simultaneously assess rates of oxidative phosphorylation and glycolysis in real-time, in live cells and in a high-throughput manner.

Introduction

Signalling via Toll-like receptors (TLRs) on dendritic cells (DCs) drives a program of activation that includes the enhanced capturing and processing of antigens for loading and presentation on major histocompatibility complex (MHC) class I and II, and the increased expression of chemokine receptors, cytokines and co-stimulatory molecules. It is not surprising that this dramatic change in the biology of DCs requires a metabolic adaptation to meet the bioenergetic and anabolic demands of this activation process. We and others have recently found that in murine DCs, triggering of TLRs is accompanied by a metabolic switch characterized by an increase in glycolysis and a complementary decrease in oxidative phosphorylation [3, 4, 14, 16, 18]. Specifically, loss of mitochondrial oxidative function was found to be a direct consequence of TLR-induced production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) that poisons the mitochondrial respiratory chain in an autocrine fashion. This forces the cells to increase glycolytic flux to maintain sufficient ATP levels [14, 76]. In addition to this bioenergetic adaption, we have recently observed that TLR engagement also triggers a rapid increase in glycolysis, preceding iNOS expression, that primarily appears to serve an anabolic role allowing DCs to express activation markers and cytokines and therefore, to acquire their full T-cell-priming potential [3].

Some of these observations regarding TLR-driven metabolic changes in DCs have been performed using traditional cellular metabolic assays that typically involve radioactivity, cell destruction and large numbers of cells [77]. Recently, Extracellular Flux (XF) Analyzers from Seahorse Bioscience have been developed that perform highly accurate real-time measurements of cellular metabolism of living cells and tissues by simultaneously quantifying rates of extracellular acidification (ECAR) and oxygen consumption (OCR) as measures of glycolysis and mitochondrial respiration, respectively. This apparatus has allowed us to gain exciting new insights in immune cell metabolism and has been shown to be instrumental in moving the field of DC metabolism forward [9]. This state-of-the-art technology offers a robust and simple high-throughput method for studying substrate utilization, mitochondrial function, and energy expenditure in a 24- or 96-well plate format, without the use of large number of cells, electrodes, dyes, radioactive materials or lysis of cells that is typical of other more laborious metabolic assays. During measurements, the XF assay cartridge is lowered, creating a temporary 7 µL microchamber with limited diffusion. In this small volume of medium, oxygen consumption and lactic acid excretion by the cells will rapidly result in significant changes in oxygen and proton concentration, which is registered by proton and oxygen-quenchable fluorophores that are embedded in the sensor (*see* Fig. 1).

In addition, as the assay is running, compounds can be injected through the four injection ports surrounding the sensor. This allows evaluation of the acute effects that compounds such as TLR ligands, metabolic substrates, activators/inhibitors of signalling pathways and other compounds of interest have on cellular metabolism and energetics. In conclusion, XF Analyzers from Seahorse Bioscience are easy to use and allow for the measurement and manipulation of metabolic pathways in real-time, helping the researcher to elucidate the involvement of metabolic processes in TLR-driven changes in DC biology. We here describe a detailed protocol of how cellular metabolism of DCs can be studied following TLR stimulation, using the 96-well format Extracellular Flux (XF^e96) Analyzer (*see* **Note 1**). We will provide one example of how changes in metabolism can be followed in real-time in response to acute TLR stimulation and one example of how mitochondrial function can be assessed in TLR-activated DCs.

Materials

1. Poly-D-lysine hydrobromide (PDL; Sigma).
2. RPMI-1640 medium powder with L-glutamine, without glucose and sodium bicarbonate (Sigma) (*see* **Note 2**).
3. 37 % HCl solution.
4. 500 mL vacuum filter/storage bottle system, 0.22 µM pore 40 cm^2 PES membrane (Corning).
5. Oligomycin (Cayman Chemical).
6. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; Sigma).
7. Rotenone (Sigma).
8. Antimycin A (Sigma).
9. DMSO.
10. XF^e96 FluxPak (Seahorse Bioscience) (*see* **Note 3**).
11. XF Calibrant (Seahorse Bioscience).
12. 10 % D-glucose (Sigma).
13. Fetal calf serum, heat-inactivated at 56 °C for 30 min (HI-FCS; Bodinco).
14. 200 µL Flextop ultra-fine point tips (VWR).
15. XF^e96 Extracellular Flux Analyzer (Seahorse Bioscience).

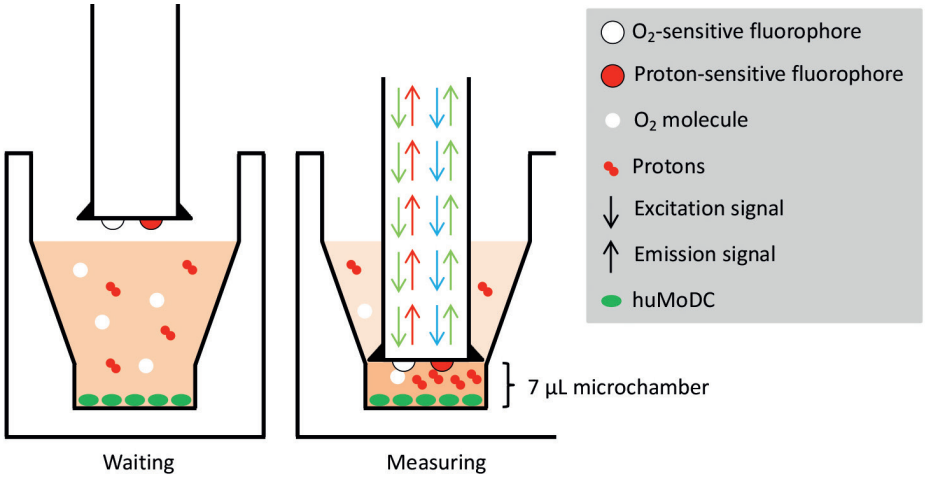

Figure 1. Schematic representation of the XF Analyzers from Seahorse Bioscience.

Methods

3.1 Preparation of the Reagents

3.1.1 Poly-D-Lysine Hydrobromide

1. Dissolve 5 mg poly-D-lysine hydrobromide (PDL) in 100 mL MilliQ H_2O to reach a concentration of 50 µg/mL.
2. Sterilize by filtration (0.2 µM) and store at −20 °C. Thawed aliquots can be stored at 4 °C.

3.1.2 Assay Media

1. Dissolve 8.4 mg RPMI-1640 medium powder with L-glutamine in 500 mL MilliQ H_2O by gentle swirling.
2. Once the powder is dissolved, add MilliQ H_2O to a total of approximately 1000 mL.
3. Adjust the pH with 37 % HCl solution to pH 7.4.
4. Sterilize the medium using a filter system.
5. To prepare 0 % FCS/XF media containing 10 mM D-glucose for use in the injection ports (*see* **Note 4**), add 0.91 mL of 10 % D-glucose to 49.09 mL of RPMI-1640 medium with L-glutamine. To prepare 5 % FCS/XF media containing 10 mM D-glucose for the cells (*see* **Notes 5 and 6**), add 0.91 mL of 10 % D-glucose and 2.5 mL of HI-FCS to 46.59 mL of RPMI-1640 medium with L-glutamine.

3.1.3 Oligomycin

1. To prepare a 1 mM stock solution, dissolve 1 mg of oligomycin in 1.26 mL DMSO. This stock needs to be diluted to 1 µM (1000×) for use in an XF assay run (*see* Table 2).
2. Prepare aliquots of 27 µL/vial and store at −20 °C.

3.1.4 FCCP

1. To prepare a 30 mM superstock, dissolve 10 mg of FCCP in 1.3 mL DMSO.
2. Subsequently, dilute 30 mM FCCP 1:10 with DMSO to generate a 3 mM stock solution. This stock needs to be diluted to 3 µM (1000×) for use in an XF assay run (*see* Table 2).
3. Prepare aliquots of 29 µL/vial and store at −20 °C.

3.1.5 Rotenone

1. To prepare a 10 mM superstock, dissolve 10 mg of rotenone in 2.5 mL DMSO.
2. Subsequently, dilute 10 mM rotenone 1:10 with DMSO to generate a 1 mM stock solution. This stock needs to be diluted to 1 µM (1000×) for use in an XF assay run (*see* Table 2).
3. Prepare aliquots of 35 µL/vial and store at −20 °C.

3.1.6 Antimycin A

1. To prepare a 10 mM superstock, dissolve 25 mg of antimycin A in 4.5 mL DMSO.
2. Subsequently, dilute 10 mM antimycin A 1:10 with DMSO to generate a 1 mM stock solution. This stock needs to be diluted to 1 µM 1000× for use in an XF assay run (*see* Table 2).
3. Prepare aliquots of 35 µL/vial and store at −20 °C.

3.2 Preparation of the XFe96 Assay Run

In brief, the general procedure for an XF assay run consists of the following steps:

1. Hydrate the XF assay cartridge.
2. Seed the cells in the cell culture plate.
3. Replace the culture medium with the assay medium.
4. Load the injection ports of the assay cartridge with the drugs/ stimuli of interest.
5. Create an assay template using XF Wave.
6. Start the calibration of the sensors in the cartridge.
7. Load the cell culture plate that contains the cells.
8. Optional: recover the cells for future cell count normalization (*see* **Note 7**).

3.2.1 Hydration of the XF Assay Cartridge

1. Place the assay cartridge upside down next to the utility plate.
2. Fill each well of the utility plate with 200 µL of calibration solution and put the cartridge back onto the utility plate, submerging the sensors in the solution.
3. Incubate for 4-24 h at 37 °C in a dry incubator without CO_2.

3.2.2 Seeding and Adherence of DCs

1. If using murine bone marrow-derived dendritic cells (BMDCs) that are cultured with GM-CSF, proceed to **Step 5**. If using any other type of DCs, coat the wells of the XF cell culture 96-well microplate with 25 µL 50 µg/mL PDL (*see* **Note 8**). Gently tap the plate to make sure that the liquid completely covers the bottom of the well.
2. Incubate with PDL for at least 1 h at 37 °C. The type of incubator does not matter at this specific step.
3. Add 175 µL sterile MilliQ H_2O to the wells, resuspend and pipet off as much liquid as possible (*see* **Note 9**).
4. Let the cell culture plate dry in a sterile flow hood for 30–60 min.
5. Culture or isolate your preferred type of DC for use in the XF assay run according to protocols described elsewhere for human monocyte-derived DCs (moDCs) [78], murine BMDCs [79] or DCs isolated from human or murine tissues [3, 80].
6. Seed the number of DCs needed to obtain a confluent mono- layer in 50 µL of the same type of culture medium in which the DCs were grown. Different DC types have different sizes and therefore, their seeding density differ (*see* Table 1).
7. Quick-spin the plate to bring all DCs to the bottom of the well.
8. Check under the microscope for a confluent monolayer.
9. Incubate the cells for 1 h at 37 °C, 5 % CO_2, 95 % humidity to allow the cells to adhere.
10. Check under the microscope for adherence.
11. Proceed to Subheading 3.2.3 to perform a mitochondrial stress test or Subheading 3.2.4 to assess in real-time metabolic changes in response to acute TLR stimulation. Of note, the assays described here are only examples of assays that can be performed using XF Analyzers (*see* **Note 10**).

Table 1. Proposed seeding densities for different DC types to reach a confluent monolayer in a XF cell culture 96-well microplate.

Source	Cells/well
Mouse bone marrow-derived, cultured with GM-CSF	70,000
Mouse bone marrow-derived, cultured with Flt3L	150,000
Mouse spleen	150,000
Human skin	200,000
Human blood	200,000
Human monocyte-derived	50,000

3.2.3 Mitochondrial Stress Test

The mitochondrial stress test allows one to interrogate the functional properties of the electric transport chain. It consists of the sequential injection of oligomycin (inhibitor of mitochondrial ATP synthase), FCCP (ionophore) and rotenone + antimycin A (inhibitors of complex one and three of the respiratory chain respectively). This allows one to assess baseline respiration (BR), oxygen consumption used for ATP production (ATP) following oligomycin injection, the maximum rate of mitochondrial respiration (MR) following FCCP injection, and non-mitochondrial respiration (NMR) following rotenone + antimycin A injection. The difference in oxygen consumption rate (OCR) between BR and MR is known as the spare respiratory capacity (SRC) and the difference in OCR after oligomycin treatment versus rotenone + antimycin A is the amount of respiration used to compensate for proton leak (PL), which is also known as uncoupling. An example of a mitochondrial stress test performed on LPS-stimulated murine BMDCs is shown in Fig. 2. The concentrations of oligomycin, FCCP and rotenone + antimycin A used in this assay can be found in Table 2.

1. Slowly add 150 µL more culture medium with or without your Toll-like receptor (TLR) ligand(s) of interest.
2. Incubate the TLR-stimulated DCs for 2 up to 48 h at 37 °C, 5 % CO_2 and 95 % humidity.
3. An hour before the XF assay run, prepare 10× working concentrations of oligomycin, FCCP and rotenone + antimycin A by diluting the drugs 1:100 in 0 % FCS/XF assay medium. For example, dilute 25 µL oligomycin in 2475 µL 0 % FCS/XF assay medium. Do not use 5 % FCS/XF assay medium for injection of compounds (*see* **Note 4**) and dilute rotenone and antimycin A together.
4. Carefully pipette off all the culture medium (*see* **Note 8**).
5. Slowly add 180 µL 5 % FCS/XF assay medium to the cells. Be especially careful with the first 50 µL (*see* **Note 8**). If the cells detach, spin-down the plate again.
6. Incubate the cells for 1 h at 37 °C in a dry incubator without CO_2 in order to remove any CO_2 dissolved in the assay medium (*see* **Note 11**).

7. Add 20 µL oligomycin to port A, 22 µL of FCCP to port B and 25 µL of rotenone + antimycin A to port C (*see* Fig. 3). Use a multichannel, special narrow tips (*see* **Item 14**, Subheading 2) and the provided loading guides to pipet the compounds of interest into the injection ports of the XF assay cartridge via a single stream (*see* **Note 12**).
8. Fill any empty injection ports of series A, B and C with 20, 22 and 25 µL of 0 % FCS/XF assay medium respectively (*see* **Note 12**).
9. Put the assay cartridge back into a 37 °C incubator without CO_2 until the start of the run (*see* Subheading 3.3).

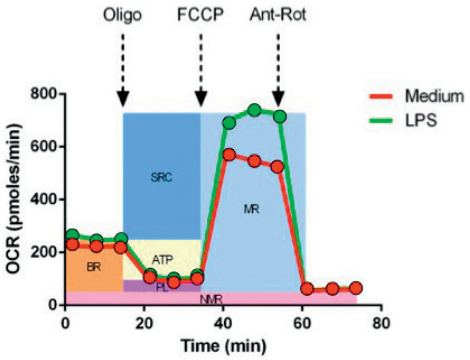

Figure 2. Murine BMDCs are stimulated with medium or LPS for 2 h and then subjected to the mitochondrial stress test. This graph suggests that short-term LPS stimulation promotes maximal respiration and spare respiratory capacity in these DCs.

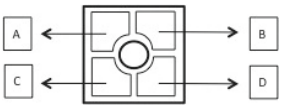

Figure 3. Schematic drawing of the four injection ports per well.

Table 2. Proposed final concentrations of the mitochondrial stress test compounds.

Oligomycin	1 µM
FCCP	3 µM
Rotenone	1 µM
Antimycin A	1 µM

3.2.4 Tracking Real-Time Metabolic Changes

DCs can also be stimulated with TLR ligands during a run in the XF Analyzer. This enables one to follow in real-time the immediate metabolic changes that are induced by TLR engagement. This can easily be combined with pre-incubations of stimulatory/inhibitory compounds to interrogate the involvement of specific signalling pathways in TLR-induced metabolic changes. An example is given in Fig. 4.

1. Slowly add an additional 150 µL culture medium.
2. Allow the cells to fully adhere and rest overnight at 37 °C, 5 % CO_2 and 95 % humidity.
3. The next day, prepare 10× working concentrations of your TLR ligand(s) and stimulatory/inhibitory compound(s) of interest in 0 % FCS/XF assay medium. Do not use 5 % FCS/ XF assay medium for injection (*see* **Note 3**).
4. Carefully pipette off all the culture medium (*see* **Note 7**).

5. Slowly add 180 µL 5 % FCS/XF assay medium to the cells. Be especially careful with the first 50 µL (*see* **Note 7**).
6. Incubate the cells for 1 h at 37 °C in a dry incubator without CO_2 in order to remove the CO_2, which was dissolved in the culture medium (*see* **Note 11**).
7. Add 20 µL of your stimulatory/inhibitory compound(s) of interest to port A and 22 µL of your TLR ligand(s) to port B (*see* Fig. 3). Use a multichannel, special narrow tips (*see* **Item 14**, Subheading 2) and the provided loading guides to pipet the compounds of interest into the injection ports via a single stream (*see* **Note 12**).
8. Fill any empty injection ports of series A and B with 20 and 22 µL of 0 % FCS/XF assay medium respectively (*see* **Note 12**).
9. Put the assay cartridge back at 37 °C in a dry incubator without CO_2 until the start of the run (*see* Subheading 3.3).

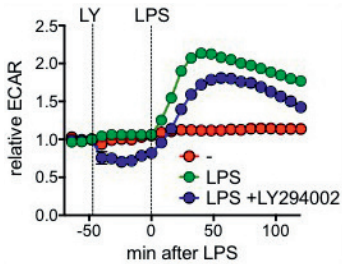

Figure 4. Murine BMDCs are stimulated with medium or LPS during a XF Assay run and the effects on extracellular acidification rate (ECAR) are assessed in real-time. Moreover, in one condition, an inhibitor of PI3K signalling (LY204002) is injected into the wells prior to LPS stimulation. This suggests that baseline glycolysis rates are partially dependent on PI3K signalling, whereas LPS-induced glycolysis is not.

3.3 Starting a XF Assay Run

A Seahorse XF Analyzer comes with a desktop computer and software (XF Wave) that is used to set up the assay template (i.e. plate layout, number and timing of injection(s), and measurement frequency and duration) and to start or cancel the XF assay run.

1. Select the 'Blank' template and click the 'Design' button.
2. Click on the vertical bar of the 'Group Definitions' tab. Define your assay conditions and then, click the 'Generate Groups' button. XF Wave will automatically generate groups based on every possible combination of assay conditions you define.

3. Click on the vertical bar of the 'Plate Map' tab. Determine the groups in the plate layout by first selecting a specific group and then, clicking on or dragging across the corresponding well(s).
4. Click on the 'Instrumental Protocol' tab. Then, click on the 'Injection Button' three times when performing the mitochondrial stress test (*see* Subheading 3.2.3) or two times when performing the real-time tracking of metabolic changes (*see* Subheading 3.2.4). For each new injection, the software will automatically select the next available port, i.e. injection 1 = port A, 2 = B, 3 = C and 4 = D.
5. A measurement typically consists of 3 min of 'mixing' and 3 min of 'measuring', repeated for 3 times (i.e. 3 cycles). Correspondingly, with one basal measurement and 3 measurements after injection, the mitochondrial stress test as described here is 72 min long (*see* Fig. 2), excluding calibration and equilibration. Longer measurement times, for example after stimulation with TLR ligands (*see* Fig. 3), can easily be achieved by increasing the number of cycles (*see* **Notes 10 and 13**). Do not forget to set the 'waiting' time at 0 min and 0 s.
6. Go to the 'Review and Run' tab to find the 'Run' button. Click 'Run'.
7. The machine will first ask to load the XF assay cartridge and the utility plate in which the sensors of the assay cartridge were hydrated (*see* Subheading 3.2.1). It is important to remove the lid from the assay cartridge and orient the cartridge and plate in a way that the blue-marked corner faces you. In this orientation, the barcode on the cartridge should not be visible.
8. Press 'Continue'.
9. The sensors in the cartridge will now be calibrated to assure the accuracy of your instrument. Equilibration occurs after calibration and ensures temperature stability before beginning your assay. Together this will take approximately 20–25 min.
10. After the calibration and equilibration, the XF Analyzer will eject the utility plate. Replace this plate with the cell culture plate containing the DCs as prepared in Subheading 3.2.3. Again, it is important to remove the lid from the plate and to orient the plate in a way that the blue marked corner faces you.
11. Press 'Continue' to start the XF assay run. Data will be displayed in real-time during the measurement.

A full tutorial on how to set up an assay template using the XF Wave software can be found on the website of Seahorse Bioscience: http://www.seahorsebio.com/resources/pdfs/user-guide-xfe- wave.pdf.

3.4 Data Analysis

Data that are obtained include ECAR and OCR as well as the raw pH and O_2 tension values. XF Wave software allows one to visualize all these parameters by creating

and exporting various graphs. Moreover, this software allows one to directly export the raw data tables to programs such as Excel or GraphPad Prism, which you can use to make your own graphs. An extensive guide on how to analysis your data using Wave can be found on the website of Seahorse Bioscience: http://www.seahorsebio.com/resources/ pdfs/user-guide-xfe-wave.pdf.

4 Notes

1. The same assays can be performed using a 24-well format. The number of cells per well in the XF^{e}24 Extracellular Flux Analyzer needs to be increased by 2.5-fold compared to the 96-well format described here (*see* Table 1). In addition, the volumes of assay media in the wells and the injection ports need to be increased by 2.5-fold.
2. The assay medium should not contain any buffering reagents, because glycolysis is determined by changes in extracellular pH.
3. An XF^{e}96 FluxPak contains equal amounts of XF assay cartridges and XF cell culture 96-well microplates. Each assay cartridge comes with its own 96-well microplate, which is referred to as the XF utility plate and is used to hydrate the cartridge sensors. Moreover, each cartridge comes with its own loading guides. The XF cell culture 96-well microplate is used for loading the cells.
4. Large proteins such as bovine serum albumin (BSA) may block the injection port.
5. FCS has some buffering capacity. The more FCS is added to the XF assay medium, the lower your ECAR readings will be.
6. FCCP concentration is dependent on the amount of protein in the XF assay medium. The higher the protein concentration, the more the effect of FCCP is quenched. In our hands, 3 μM FCCP works well with the amount of proteins present in 5 % FCS/assay medium to induce maximum respiration and glycolysis (*see* Table 2).
7. Generally, DCs are fully differentiated, non-dividing cells. However, in the case observed differences in metabolism are suspected to be due to differences in cell density rather than inherent differences in metabolism, one can do a normalization after the XF Assay run. For example, one can normalize the obtained metabolic readouts based on protein quantification methods such as the bicinchoninic acid (BCA) assay. The XF Wave software has a feature to enter such data and automatically perform normalization.
8. During measurement, the XF assay cartridge sensors only measure the bottom 7 μL of the XF cell culture 96-well microplates. Therefore, your cells need to be at the bottom of the well and immobilized. XF cell culture microplates are made of polystyrene and are tissue culture-treated, but in our experience, all DCs, except murine bone marrow-derived DCs cultured with GM-CSF, require an additional plate bound substrate such as PDL for proper adherence. Note that adherence by PDL may cause some activation of DCs.
9. It is preferable to wash with unbuffered solutions.

10. Other regularly performed assays include the beta-oxidation assay and the glycolysis stress test. Reagents for these assays are available through Seahorse Bioscience. Moreover, the flexibility of XF Analyzers allows one to easily deviate from standard protocols in order to address specific metabolic questions. For example, the presence of certain nutrients in the medium, the nature of the compounds, the timing of injections and the duration of measurements can be adjusted.
11. CO_2 reacts with H_2O to form HCO_3^- + H^+, which acidifies the medium and results in incorrect ECAR readings.
12. XF Analyzers use compressed air to inject compounds from the ports into the wells. Moreover, series of injection ports are linked (i.e. all A ports, all B ports, etc.). Therefore, each series of injection ports must contain the same amount of volume for the injections to work.
    a. The combination of these tips (*see* **Item 14**, Subheading 2) and the loading guides allows on to insert the tip into the injection port at a specific depth. Too high and droplets may stick at the top of the well. Too low and the solution may be pipetted through the injection port.
    b. Dispense the solution via a single stream. Otherwise, droplets may stick at the end of the tip.
    c. Do not tap the XF assay cartridge in order to get the solution at the bottom of the injection port. The XF assay cartridge is fragile and tapping may cause leaking of the injection ports.
13. We experienced that reliable ECAR and OCR reads can be obtained up to 6 h into a run. After 6 h, XF assay medium becomes too acidified.

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