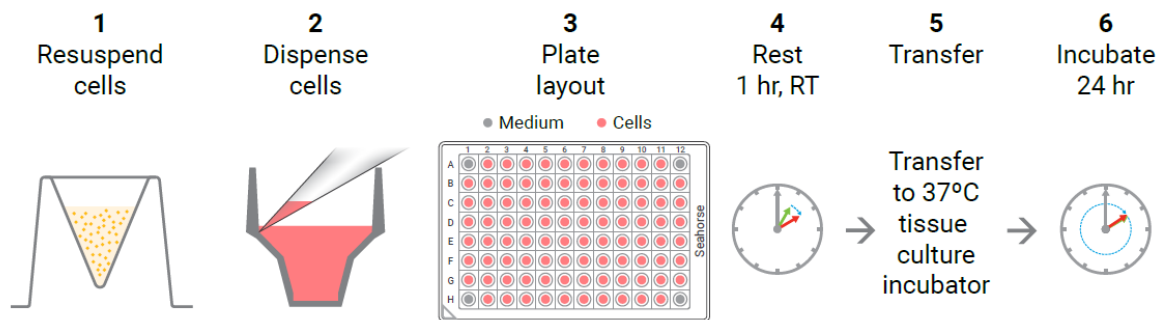


Seahorse XF96 Protocol – adapted from Agilent

Seeding cells in XF96 Cell Culture Microplates

1. Harvest and re-suspend the BMDMs to the desired concentration to seed in 80 μL of growth medium (~20,000 cells per well is optimal)
2. Seed 80 μL of cell suspension per well (as shown in the figure below). Do not seed cells in the background correction wells (A1, A12, H1, H12).
3. Allow the plate to rest at room temperature in the cell culture hood for 30-60 min. Afterwards, transfer the plate to a humidified 37°C CO₂ incubator.
4. Allow the cells to settle overnight, and add 120 μl medium the following day (or directly perform an efferocytosis experiment by adding ACs in 20 μL /well)

Basic Cell Seeding Workflow



Performing the XF96 Seahorse experiment

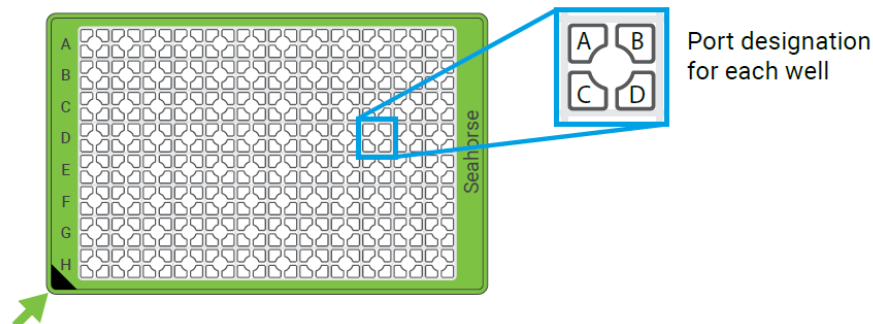
One day after seeding the differentiated cells into the Seahorse plate, the experiment can be performed:

1. One night in advance, add Seahorse XF Calibrant (200 μL /well) to the sensor cartridge, and put this in a 37°C CO₂-free incubator (and humidify the incubator, to avoid evaporation).
2. Prepare Seahorse medium, one day in advance or the day of the experiment. Per 50 ml Seahorse medium add 0.5 mL of L-glutamine (200 mM) and optionally 0.5 ml of sodium pyruvate (100 mM). Warm this medium to 37°C before using.
3. On the day of the experiment, remove the regular medium from the Seahorse plate, wash once with 150-200 μL Seahorse medium, and then add 180 μL fresh Seahorse medium for the assay.
4. Put the cells in a 37°C CO₂-free incubator 30-60 min before starting the run.
5. In the meantime, take the cartridge out of the incubator, and load it with the necessary compounds (see figure and example calculations below) diluted in Seahorse medium.
6. Open the Seahorse run template (see instructions below) and click START. Next, put in the cartridge for calibration (without the lid, this takes approximately 30 min). After this, you can put the cell plate in (without lid) and run the assay.

	Mito stress test	Glycolysis stress test
Starting assay volume	180 μ L	180 μ L
Injection Port A	20 μ L Oligomycin	20 μ L Glucose
Injection Port B	22 μ L FCCP	22 μ L Oligomycin
Injection Port C	25 μ L Rotenone and Antimycin A	25 μ L 2-DG

- All compounds should be injected in a 10x stock solution
- Oligomycin 20 μ M injection to final concentration of 2 μ M (for both MST and GST)
- FCCP 15 μ M injection to final concentration of 1.5 μ M
- Rotenone and Antimycin A 20 μ M injection (of both) to a final concentration of 2 μ M
- Glucose 200 mM injection to a final concentration of 20 mM
- 2-DG 1 M injection to a final concentration of 100 mM

1. Port A: 20 μ l
2. Port B: 22 μ l
3. Port C: 25 μ l
4. Port D: 28 μ l



Calculations for the XF96 Seahorse experiment

1. Macrophages can be incubated with ACs prior to the experiment. Add ACs in a 5:1-10:1 AC:macrophage ratio (so 100,000-200,000 ACs per well) in 20 μ L medium.
2. For short-term incubation with ACs (~1h), add ACs for 40 min, then wash them off with Seahorse medium, incubate for another 30 min and then run the assay (by the time the measurement starts it will be approximately 1h post-efferocytosis).
3. For half a plate Mito Stress Test:
 - a. 1.5 ml Seahorse medium + 1.5 μ l oligomycin stock
 - b. 1.5 ml Seahorse medium + 1.5 μ l FCCP stock
 - c. 1.5 ml Seahorse medium + 1.5 μ l rotenone + 1.5 μ l antimycin A stock
4. For half a plate Glycolysis Stress Test:
 - a. 1.5 ml Seahorse medium + 1.5 μ l oligomycin stock
 - b. 1.5 ml Seahorse medium + 54 mg glucose
 - c. 1.5 ml Seahorse medium + 246 mg 2-DG

Prepare the plate layout in Wave software

- **‘Group definitions’**
 - Under injection strategies, fill in the different compounds that are injected in your experimental setup
 - Fill in pretreatments if you have pretreated your cells
 - Generate groups, and remove the generated groups that do not match your experimental setup
- **‘Plate map’**
 - Assign the groups to the corresponding wells on the plate
 - Assign the background wells
- **‘Instrumental protocol’**
 - Leave the standard settings as they are, but adjust your injection strategy (see example below)
 - For each filled injection port, you have to assign a separate injection strategy. Select the port and check the measurement details and group summary
- **‘Review and run’**
 - Fill in the project name
 - Adjust the well volume to 180 μL

Protocol Start

1. *Calibrate Probes*
2. *Equilibrate*
3. *Loop 3 times*
 - 4. (1) *Mix for 3 Min. 0 sec.*
 - 5. *Time delay of 2 Min. 0 sec.*
 - 6. (1) *Measure for 2 Min. 0 sec.*
7. *Loop End*
8. *Inject Port A*
9. *Loop 3 times*
 - 10. (2) *Mix for 3 Min. 0 sec.*
 - 11. *Time delay of 2 Min. 0 sec.*
 - 12. (2) *Measure for 2 Min. 0 sec.*
13. *Loop end*
14. *Inject Port B*
15.

Protocol End