



POTENTIATING THE ITALIAN CAPACITY FOR STRUCTURAL BIOLOGY SERVICES IN INSTRUCT-ERIC

PROTOCOL FOR OPERATING A NMR BIOREACTOR FOR REAL-TIME IN-CELL NMR

IO2.2.2: Advanced Bioreactor for in-cell NMR studies

Activity 2.2: Expanding the mammalian expression platform for in-cell NMR

WP leader: Prof. Marco Fragai

Period: B12 (September - October 2024)

Author(s): Prof. Marco Fragai, Dr. Enrico Luchinat (CERM-UniFi)

Call for proposals MUR n. 3264 in date 28/12/2021

Mission 4 – Education and Research Component 2: from research to business

Investment 3.1 Fund for the realisation of an integrated system of research and innovation infrastructures

Action 3.1.1 Creation of new research infrastructures, strengthening of existing ones and their networking for Scientific Excellence under Horizon Europe

RI : INSTRUCT-ERIC – Integrated Structural Biology Infrastructure

ESFRI domain: Health and Food

RI at high priority

Funding Admission DD MUR n. 115 in date 21/06/2022

Starting Date: 1 November 2022

Duration of the project: 30 month

ID Proposal: IR0000009

CUP: B53C22001790006

Protocol for operating a NMR bioreactor for real-time in-cell NMR

This protocol describes the setup of an NMR bioreactor to keep encapsulated human cells viable for up to 72 hours for time-resolved in-cell NMR applications, such as monitoring intracellular protein–ligand interactions in real time in HEK293T cells.

Materials

- D₂O (Merck 453366)
- DMEM, high glucose (Life Technologies 10313-021)
- DMEM, high glucose, powder (Merck D5648)
- FBS (Life Technologies 10270)
- HCl (Merck 30721)
- L-glutamine 200 mM (Life Technologies 25030)
- Low-gelling agarose, powder (Merck A4018)
- NaHCO₃, powder (Carlo Erba 478537)
- PBS (Life Technologies 10010)
- Penicillin–streptomycin 10,000 U/ml (Life Technologies 15140-122)
- NaOH, pellets (Merck 30620)
- Trypan Blue solution 0.4% (wt/vol) (Merck T8154)
- Trypsin–EDTA 0.05% (wt/vol) (Life Technologies 25300-054)
- 100 µm cell strainer (Corning 431752)
- 0.22 µm syringe filter (Merck SLGL0250S)

Equipment

- NMR Spectrometer equipped with a 5 mm CryoProbe.
- InsightMR 2.0 flow unit (Bruker)
- Pumps: several types can be employed, for example:
 - P-920 pump module from ÄKTA FPLC (Cytiva)
 - Reglo ICC Digital Pump, 3-channel (Ismatec)
 - µDispense dosing system equipped with a micro annular gear pump and an integrated control board (HNP Mikrosysteme)

1. Reagent and solution setup

1.1 Prepare complete DMEM

1.1.1 Add 5 mL L-glutamine 200 mM, 5 mL penicillin–streptomycin 100x and 50 mL fetal bovine serum (FBS, 10% vol/vol final concentration) to 440 mL DMEM.

NOTE: this solution can be stored at 4 °C for 1 month.

1.1.2 Prepare agarose solution

1.1.3 Dissolve 150 mg low-gelling agarose in 10 mL phosphate buffered saline (PBS) at 85 °C to obtain a 1.5% (w/v) solution.

1.1.4 Sterilize by filtration with a 0.22 µm filter.

1.1.5 Prepare 1 mL aliquots of agarose solution in 1.5 mL capped tubes.

1.1.6 Store at 4 °C.

1.2 Prepare the bioreactor medium

1.2.1 For 1 L of medium, dissolve 13.4 g DMEM powder, 0.8 g NaHCO₃ and 30 mL D₂O and bring to 1 L with ultrapure H₂O.

NOTE: depending on the application, the required final volume may differ (e.g. for 500 mL medium, use 6.7 g powder DMEM, etc.)

1.2.2 Measure the pH using a pH-meter and if needed adjust to 7.4 by adding HCl. NOTE: typically, the initial pH is very close to 7.4.

1.2.3 Filter the solution with a vacuum driven sterile filter in a sterile 250-/500-/1000-mL glass bottle.

1.2.4 In the laminar flow hood, add 2% FBS and 1% penicillin–streptomycin 100x solution.

1.2.5 Close the bottle with a custom-made cap containing two holes, one for the inlet (ETFE tubing o.d. = 1/16", i.d. = 1.0 mm) that will be connected to the pump and the other fitted with a 0.22 µm PTFE syringe filter.

2. Bioreactor setup

NOTE: the flow unit should be already cleaned (if not, perform step 4.2).

2.1 Assemble the flow unit. Refer to the flow unit operating instructions for the correct assembly. If a filter mesh is already in place, go to step 2.4.

2.2 Create a nylon mesh filter by cutting a ~4 mm circular shape from a 100 µm cell strainer. The cut can be done by hand or using a leather hole puncher.

2.3 With the help of a sewing needle, punch a hole in the center of the mesh, transfer to the end of the inlet tubing of the bioreactor (PEEK orange tubing o.d. = 1/32", i.d. = 0.5 mm) and slide it up to touch the outlet of the flow chamber.

2.4 Close the system by inserting a clean flow NMR tube, which will be later replaced with the one containing the cells.

2.5 Set the water bath connected to the flow unit temperature control to 37 °C. Place the reservoir bottle in the water bath.

2.6 Connect the ETFE tubing of the reservoir bottle to the pump.

2.7 Turn the bioreactor valve to 'bypass' and prefill the pump with medium.

2.8 Turn the bioreactor valve to 'flow' and prefill the bioreactor with medium at 0.1 mL/min.

3. Preparation of the cell sample

3.1 Collect the cells from the CO₂ incubator

- 3.1.1 Take a T75 flask of transfected HEK293T cells from the CO₂ incubator and remove the spent medium.
- 3.1.2 Wash the cells twice with 7 mL (each) of PBS at room temperature (~20 °C).
- 3.1.3 Detach the cells with 2 mL of trypsin-EDTA: incubate them for 5 min at room temperature to detach the cells.

NOTE: trypsin may take slightly longer to detach transfected cells. If necessary, incubate the cells at 37 °C.
- 3.1.4 Inactivate trypsin with 20 mL of complete DMEM, thoroughly resuspend the cells by pipetting up and down and transfer them in a 50 mL falcon tube.
- 3.1.5 Centrifuge the cells at 800 g for 5 min at room temperature, and discard the supernatant.
- 3.1.6 Wash the cells with 10 mL of PBS at room temperature to remove the residual medium.
- 3.1.7 Centrifuge the cells at 800 g for 5 min at room temperature, and discard the supernatant.
- 3.1.8 Transfer the cells to a 1.5 mL capped tube.

3.2 Embed cells in agarose threads

- 3.2.1 Melt one aliquot of solidified agarose at 85 °C in a water bath and subsequently keep it in solution at 37 °C in a thermoblock.

3.2.2 With a Pasteur pipette, fill the bottom of the flow unit NMR tube with 60-70 μL 1.5% agarose gel and place it in ice. This will create a ~5 mm-high bottom plug that allows placing the cell sample within the active volume of the ^1H NMR coil.

NOTE: only make the bottom plug if the PEEK orange inlet tubing ends >5 mm from the bottom of the tube. DO NOT make the bottom plug if the inlet reaches the bottom of the tube. To adjust the position of the inlet tubing, refer to the InsightMR 2.0 manual.

3.2.3 Heat up at 37 °C for 15–20 s in the thermoblock the pellet of cells obtained in step 3.1.8.

3.2.4 Resuspend cells in 450 μL of agarose solution, carefully avoiding the formation of bubbles.

3.2.5 Aspirate the cell–agarose suspension into a ~30 cm-long chromatography PEEK tubing (i.d. = 0.75 mm) connected to a 1 mL syringe.

NOTE: before aspiration, the tubing and the dead volume of the syringe should be prefilled with PBS at room temperature to avoid the formation of bubbles. The length of the tubing is not critical.

3.2.6 Let the tubing cool down at room temperature for 2 min.

3.2.7 Prefill the flow unit NMR tube with 100 μL PBS at room temperature.

3.2.8 Cast threads of cells embedded in agarose into the flow unit NMR tube by gently pushing the syringe.

NOTE: to fill the NMR tube homogenously, start by placing the end of the PEEK tubing at the bottom of the NMR tube and proceed towards the top while slowly swinging left-right.

3.2.9 Repeat steps 3.2.5, 3.2.6 and 3.2.8 until all the cell-agarose suspension has been cast.

3.3 Insert the cells in the bioreactor

3.3.1 Remove the empty NMR tube from the flow unit and increase the flow rate to 1 mL/min for a few minutes, to remove residual gas bubbles in the inlet tubing.

3.3.2 Set the flow rate to 0.2 mL/min and insert the NMR tube containing the cells by pushing it upwards slowly but steadily.

NOTE: the active flow of medium avoids the backflow of tube content through the inlet, that would otherwise occur during the insertion.

4. Bioreactor operation and cleaning

4.1 Bioreactor operation during the NMR experiment

4.1.1 Set the temperature in the NMR spectrometer to 310 K.

4.1.2 Insert the flow unit in the spectrometer.

4.1.3 Supply the bioreactor medium at a flow rate of 0.1 mL/min for the whole duration of the in-cell NMR experiments.

4.2 Bioreactor clean-in-place

4.2.1 Clean the flow unit by flowing at 1 mL/min of 0.2 M sodium hydroxide (NaOH) for at least 30 min, followed by sterile-filtered ultrapure water for >2 h.

4.2.2 Clean and autoclave the reservoir bottle and tubing assembly after each run.

5. NMR experiments

5.1 Setup the desired NMR experiments. Depending on the application, either ^1H , ^1H - ^{13}C , ^1H - ^{15}N or ^{19}F spectra can be recorded.

5.2 Real-time NMR spectra acquisition

5.2.1 Once the bioreactor is inserted in the NMR spectrometer, wait a few minutes to allow the exchange of the medium.

NOTE: this process is easily monitored from the appearance of the lock signal as the PBS is replaced with medium containing 3% D₂O.

5.2.2 Adjust the matching and tuning of the $^1\text{H}/^{13}\text{C}/^{15}\text{N}/^{19}\text{F}$ channels, shim the magnet and calculate the ^1H 90° hard pulse length.

5.2.3 Adjust the ^1H power levels in each pulse sequence according to the ^1H hard pulse.

5.2.4 Record a first zgesgp ^1H spectrum to check the sample content and the field homogeneity.

5.2.5 Queue the desired type/number of NMR experiments in the acquisition spooler.

NOTE: the zgesgp spectra are only used to control the state of the sample and the field homogeneity, therefore they can be either skipped or recorded less frequently.

6. Trypan Blue Test

6.1 Recover the NMR tube content with a Pasteur pipet, and transfer the agarose threads to a 1.5 mL capped tube.

6.2 Remove the residual medium by rinsing the agarose threads with 600 μL of PBS and centrifuge them at

4000 g for 1 min at room temperature. Discard the supernatant.

6.3 Add 250 μ L of PBS and 50 μ L of 0.4% Trypan blue solution.

6.4 Incubate for 2 min with continuous pipetting

6.5 Wash twice with 600 μ L of PBS discarding the supernatant.

6.6 Place a few agarose threads on a microscope slide and chop them with razor blades to create small slices of gel. Select the thinnest slices (thickness < 0.4 mm, ideally ~0.2 mm) for the analysis.

6.7 Transfer the gel slices into a self-made cell counting chamber consisting of two glass slides spaced by three layers of parafilm (~0.4 mm total thickness) on each side.

NOTE: a Burkner chamber could also be used, however the gel slices thicker than the chamber height (0.1 mm) would be squeezed, rupturing the embedded cells.

6.8 Acquire images of cells inside the agarose and count white and blue cells.

6.9 Calculate cell viability as (total cells - blue cells)/total cells.