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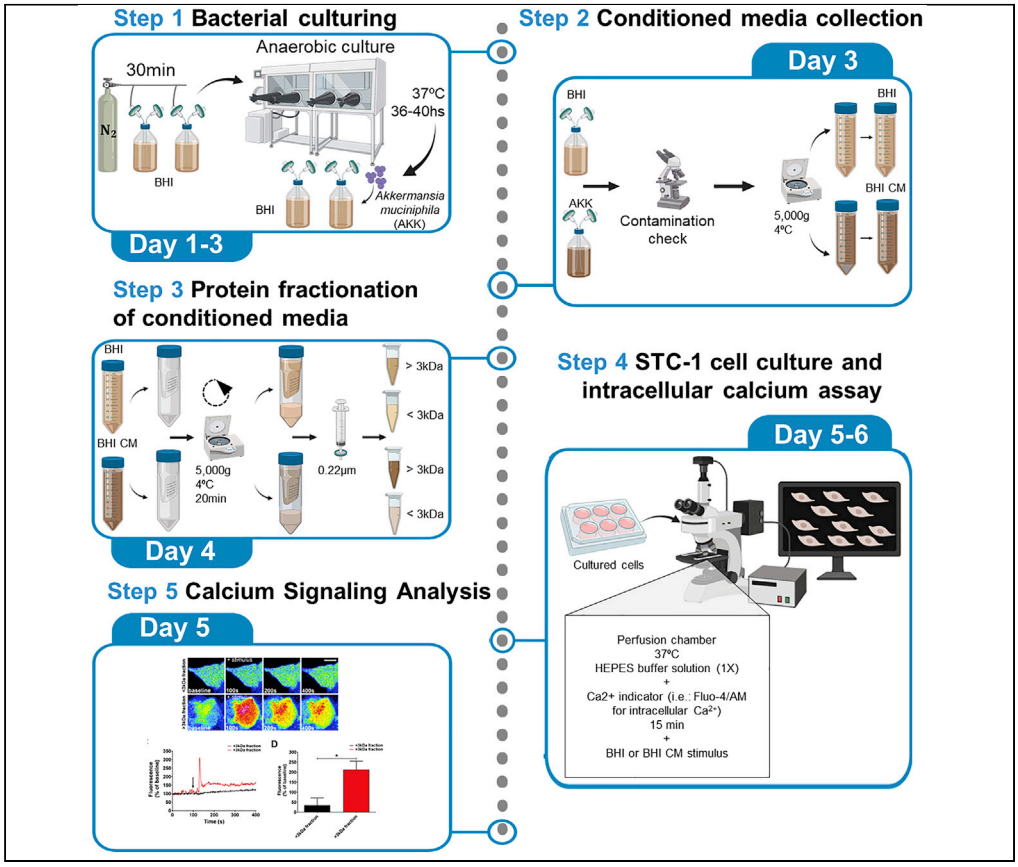
Protocol for rapid obtention and fractionation of anaerobic bacterial conditioned media to study calcium signaling in enteroendocrine cells



Gut microbiota influences neurodevelopment, behavior and contributes to neurodegenerative disorders. One possible mechanism is the direct modulation of calcium (Ca^{2+}) signaling and protein homeostasis in enteroendocrine cells (EECs), a component of the gut epithelium. Here, we present a protocol to isolate fractions of conditioned media (CM) from the anaerobic bacteria *Akkermansia muciniphila* and the utilization of this CM to monitor Ca^{2+} fluctuation in EECs by imaging. This protocol can be adapted and applied to various bacterial cultures and cell types.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for rapid obtention and fractionation of anaerobic bacterial conditioned media

Protocol can be applied to different anaerobic bacterial strains

Isolated media can be used to study Ca^{2+} signaling in several mammalian cells

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Protocol for rapid obtention and fractionation of anaerobic bacterial conditioned media to study calcium signaling in enteroendocrine cells

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SUMMARY

Gut microbiota influences neurodevelopment, behavior and contributes to neurodegenerative disorders. One possible mechanism is the direct modulation of calcium (Ca^{2+}) signaling and protein homeostasis in enteroendocrine cells (EECs), a component of the gut epithelium. Here, we present a protocol to isolate fractions of conditioned media (CM) from the anaerobic bacteria *Akkermansia muciniphila* and the utilization of this CM to monitor Ca^{2+} fluctuation in EECs by imaging. This protocol can be adapted and applied to various bacterial cultures and cell types.

For complete details on the use and execution of this protocol, please refer to Amorim Neto et al. (2022).

BEFORE YOU BEGIN

Parkinson's disease (PD) is a neurodegenerative condition featured by motor dysfunction, death of midbrain dopaminergic neurons and accumulation of α-synuclein (αSyn) aggregates. Besides the characteristic motor symptoms, PD patients show gastrointestinal dysfunction and αSyn aggregates in the gut even before Central Nervous System pathology, indicating a possible route of disease onset and spread through the gut-brain axis (Braak et al., 2003a, 2003b; Cersosimo et al., 2013). Many reports have shown that individuals with PD display an imbalanced gut microbiome (dysbiosis) (Heintz-Buschart et al., 2018; Hill-Burns et al., 2017; Keshavarzian et al., 2015) in which commensal bacteria (e.g., phylum Firmicutes) are reduced while pathogenic Gram-negative bacteria (*Proteobacteria sp*, *Enterobacteriaceae sp*, *Escherichia sp.*) and mucin-degrading members of the family Verrucomicrobiaceae, such as *Akkermansia muciniphila*, are increased (Hill-Burns et al., 2017; Keshavarzian et al., 2015; Li et al., 2017; Scheperjans et al., 2015; Unger et al., 2016). Since their discovery, the enteroendocrine cells (EECs) emerged as an important connection element of the gut-brain axis as they perform synapses with enteric neurons, express αSyn, and can develop αSyn pathology (Chandra et al., 2017; Kaelberer et al., 2020; Rodrigues et al., 2022). In addition, since these cells face the gut lumen and are in direct contact with the gut microbiome, they are constantly subjected to its stimulation and modulation. Therefore, understanding how the secreted bacterial elements can interfere with protein homeostasis in EECs is a new path of investigation to understand the contribution of these cells to PD pathology. This protocol describes the detailed obtention of the conditioned-medium (CM) from the anaerobic microorganism *A. muciniphila* which is known to modulate intracellular calcium (Ca^{2+}) signaling in EECs and induce αSyn pathology in the same cell model (Amorim Neto et al., 2022). In addition, we explain here how this CM can be used to monitor Ca^{2+} signaling in EECs under confocal live imaging. This protocol can be adapted and applied to various anaerobic bacterial cultures and cell types.

Akkermansia muciniphila cultivation in an anaerobiosis chamber

Timing: 2 days

1. Prepare 300 mL of Brain Heart Infusion (BHI) medium according to the proportions indicated by the manufacturer (see at BHI media preparation) and adjust the pH to 7.4 ± 0.2.
   a. Aliquot the medium in 50 mL portions into a bottle suitable for sterilization by moist heat (autoclave, 121°C, 15 min).

   ⚠ CRITICAL: at the bottles cap exit there must be at least two properly sealed air exit systems containing an attached 0.22 μm filter.

   b. Next, couple the bottles containing the sterile media into a nitrogen (N_2) gas injection system and keep it on for 30 min.

   ⚠ CRITICAL: The connection between the nitrogen gas exit and the culture medium bottles must be mediated through the 0.22 μm filter to avoid contamination.

   c. Clean the surfaces inside the anaerobiosis chamber with 70% ethanol, promote gas exchange through the anaerobiosis gas mixture (0% O_2, 5% H_2, 10% CO_2 e 85% N_2), and set the temperature to 37°C.
      i. Transfer the bottles into the chamber properly closed.
      ii. Keep them for about 12 h and observe if the liquid remains translucent, which will indicate the absence of contamination.
      iii. If necessary, with the aid of sterile utensils, remove 10 μL of medium and add it to a slide covered with a glass coverslip and check for non-contamination under light microscopy.
2. Next, 100 μL of *Akkermansia muciniphila* previously cryopreserved in BHI medium should be added to one of the bottles containing BHI to activate *Akkermansia's* metabolism. The other bottle will be used as control (BHI unconditioned media).
   a. Keep this first inoculum inside the anaerobiosis chamber for 24 h at 37°C.

   ⚠ CRITICAL: before performing the second inoculum, mount a slide and coverslip containing 10 μL of the bacterial suspension obtained in the previous item (step 2a) and observe under light microscopy to check for contamination.

Centrifugal Filter Units membrane preparation

Timing: 4 h

3. Organize a biological safety cabinet to work in a sterile environment.
4. Prepare an ice bath.

   Note: if the conditioned and unconditioned media have been frozen at −80°C, remove them from the bio freezer and place them on an ice bath until they are completely thawed (the defrosting time is variable). Then gently shake the tubes by hand and turn them upside down to homogenize the medium.

5. Adjust the setting of the 50 mL Falcon tube centrifuge to 5,000 g at 4°C, 15 min and wait until the temperature cools down.
6. Wash Centricon® 3kD Plus-70 Centrifugal Filter Units membranes to remove the glycerol (hereafter abbreviated to "centricon" only).
   a. Handle the centricon only inside the biological safety cabinet.

b. Add sterile ultrapure water to the top of the centricon until the membrane is complete and centrifuge under the conditions of step 5 until all the water has passed through the membrane.
c. Discard the water that was retained in the lower portion of the centricon and rinse it with sterile type I water.
d. Repeat steps 6b and 6c three times.

Pause point: at this step, the washed centricons can be stored at refrigeration temperature (4°C). For this purpose, the lower and upper portion of the centricon must be filled with sterile type I water and the entire membrane must be submerged until the day of use.

⚠ CRITICAL: pay attention to the turbidity of the water in the centricons as mold contamination may occur. In this case, the centricon must be discarded. For long term preservation, 0.02% sodium azide (w/v) can be added.

Optional: If you have centrifuges with greater rotational power, such as fixed-angle rotor centrifuges, this protocol can be adapted for their use. Under this condition, the time required for conditioned medium concentration and fractionation and the initial centricon's cleaning will be reduced.

STC-1 cell culture for intracellular Ca^{2+} signaling assay

Timing: 48 h plus

7. Add a sterile glass coverslip/well in a 6-well plate suitable for adherent cell culture.
8. Pipette 3 × 10^5 STC-1 cells per 2 mL of DMEM medium supplemented with 10% FBS and 1% PSA and incubate at 37°C with atmosphere regulated to 5% CO_2, at 90%–95% humidity for 48 h or until the wells reach 80% confluence on the plate.
   a. Change the medium in the wells every 24 h.

Optional: Cells can also be directed plated on 35-mm petri dishes with glass bottom proper for imaging under confocal microscopy.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Akkermansia muciniphila	DSMZ	DSM 22959, Type strain
Chemicals, peptides, and recombinant proteins		
Brain & Heart Infusion Broth (BHI)	BD	#237500
Fluo-4/AM	Thermo Fisher Scientific	F14201; CAS#: 273221-67-3
DMEM	Gibco	#11885084
Fetal bovine serum (FBS)	Gibco	#16140071
Penicillin/Streptomycin antibiotics (PSA)	Gibco	#15140122
NaCl	Sigma-Aldrich	#S9888
KCl	Sigma-Aldrich	#P3911
$MgSO_4$	Sigma-Aldrich	#M7506
NaH_2PO_4	Sigma-Aldrich	#S0751
$CaCl_2$	Sigma-Aldrich	#C4901
EGTA	Sigma-Aldrich	#324626
Dextrose	Sigma-Aldrich	# G7021
Hepes	Sigma-Aldrich	#H3375

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
STC-1	ATCC	(Cat# CRL-3254, RRID: CVCL_J405)
Software and algorithms		
Image J	(Schneider et al., 2012)	https://imagej.nih.gov/ij/
Graphpad Prism 8	GraphPad Software, Inc.	https://www.graphpad.com/scientific-software/prism/
Other		
Amicon Centricon® 3kD Plus-70 Centrifugal Filter Units	Millipore	#UFC900308
Millex-GP Syringe Filter Unit, 0.22 µm	Millipore	SLGP033RS
Syringe with Luer-Lok™ nozzle 5 mL (thread type)	BD	#990175

MATERIALS AND EQUIPMENT

HEPES buffer solution		
Reagent	Final concentration	Amount
NaCl	142.2 mM	3.8 g
KCl	5.4 mM	0.175 g
$MgSO_4$	0.8 mM	0.1235 g
NaH_2PO_4	1.0 mM	0.0815 g
$CaCl_2$	1.0 mM	0.0925 g
Dextrose	5.6 mM	0.45 g
HEPES	10 mM	2.35 g
ddH_2O	n/a	Up to 500 mL
Total	**n/a**	**500 mL**

Add 250 mL of ddH_2O to a beaker under stirring and add the reagents in the order they appear in the table. At the end of the weighing, adjust the pH solution to 7.4 with 0.5 M KOH or HCl and make up to a final volume of 500 mL with ddH_2O. The solution can be prepared up to 3 days in advance and should be stored at 4°C for up to 15 days (Fonseca et al., 2018).

HEPES Ca^{2+}-free buffer		
Reagent	Final concentration	Amount
NaCl	142.2 mM	3.8 g
KCl	5.4 mM	0.175 g
$MgSO_4$	0.8 mM	0.1235 g
NaH_2PO_4	1.0 mM	0.0815 g
EGTA	1.0 mM	0.190 g
Dextrose	5.6 mM	0.45 g
HEPES	10 mM	2.35 g
ddH_2O	n/a	Up to 500 mL
Total	**n/a**	**500 mL**

Add 250 mL of ddH_2O to a beaker under stirring and add the reagents in the order they appear in the table. At the end of the weighing, adjust the pH solution to 7.4 with 0.5 M KOH or HCl and make up to a final volume of 500 mL with ddH_2O. The solution can be prepared up to 3 days in advance and should be stored at 4°C for up to 15 days. (Fonseca et al., 2018).

STEP-BY-STEP METHOD DETAILS

Akkermansia muciniphila cultivation in an anaerobiosis chamber

Timing: 42 h

This step describes the *Akkermansia muciniphila* incubation period and its growth rate appropriate for media conditioning.

1. Add 100 μL of the first inoculum, where the bacteria were unfrozen and metabolically activated at 37°C, in 2 flasks of BHI medium and keep them in an anaerobiosis chamber at 37°C. From these flasks, *Akkermansia muciniphila* conditioned medium (BHI CM) will be collected.
2. Under the same conditions keep another 2 flasks containing only BHI that will be used later as negative controls for the experiments (BHI).
3. Keep all bottles incubated for 36–40 h until bacterial growth reaches an OD_{600} = 0.6. This information can be obtained by using a spectrophotometer. BHI unconditioned media should be used as blank.

⚠ CRITICAL: Before moving on to the next step, mount a slide and coverslip containing 10 μL of the bacterial suspension or unconditioned media obtained in the previous item (steps 1 and 2) and observe under light microscopy to check for contamination in both flasks.

Optional: Different supplementations can be used, such as adding mucin or other compounds to the bacterial culture medium. One should always remember to add them in equal proportion to the negative control flasks where the bacteria will not be inoculated.

Akkermansia muciniphila conditioned media collection

Timing: 30 min

This step describes how the planktonic bacterial cells should be separated from the supernatant and the conditioned medium collected.

4. Prepare an ice bath.
5. Organize a biological safety cabinet to work in a sterile environment.
6. Adjust the setting of the 50 mL Falcon tube centrifuge to 5,000 g at 4°C and wait until the temperature cools down.
7. Individually transfer the bacterial suspension and the pure BHI medium into sterile 50 mL Falcon tubes. Identify them properly and immediately store them in an ice bath.
8. Then position them alternately in the centrifuge and centrifuge at 5,000 g, 4°C for 10 min.
9. Within a biological safety cabinet, sterilely collect the supernatant and transfer it to a new sterile 50 mL Falcon tube. Also collect the pure BHI medium in the same way.
10. Discard the bacterial pellet.

Note: If necessary, repeat centrifugation step 8 to completely decant the planktonic *Akkermansia* cells.

Pause point: At this step, the conditioned and unconditioned media can be stored at −80°C until the time of media fractionation and concentration.

Fractionation of *Akkermansia muciniphila* conditioned media

Timing: 1 day

This step describes the process of concentrating of BHI CM and BHI. In the case of BHI CM, the > 3 KDa fraction is rich in proteins produced by *Akkermansia* while the < 3 KDa fraction is very reduced in proteins. This distinction can be elucidated using Nuclear Magnetic Resonance (NMR) approach (Figure 1A).

11. Inside a biological safety cabinet, sterilely and individually transfer the BHI CM or BHI to the centricon top.

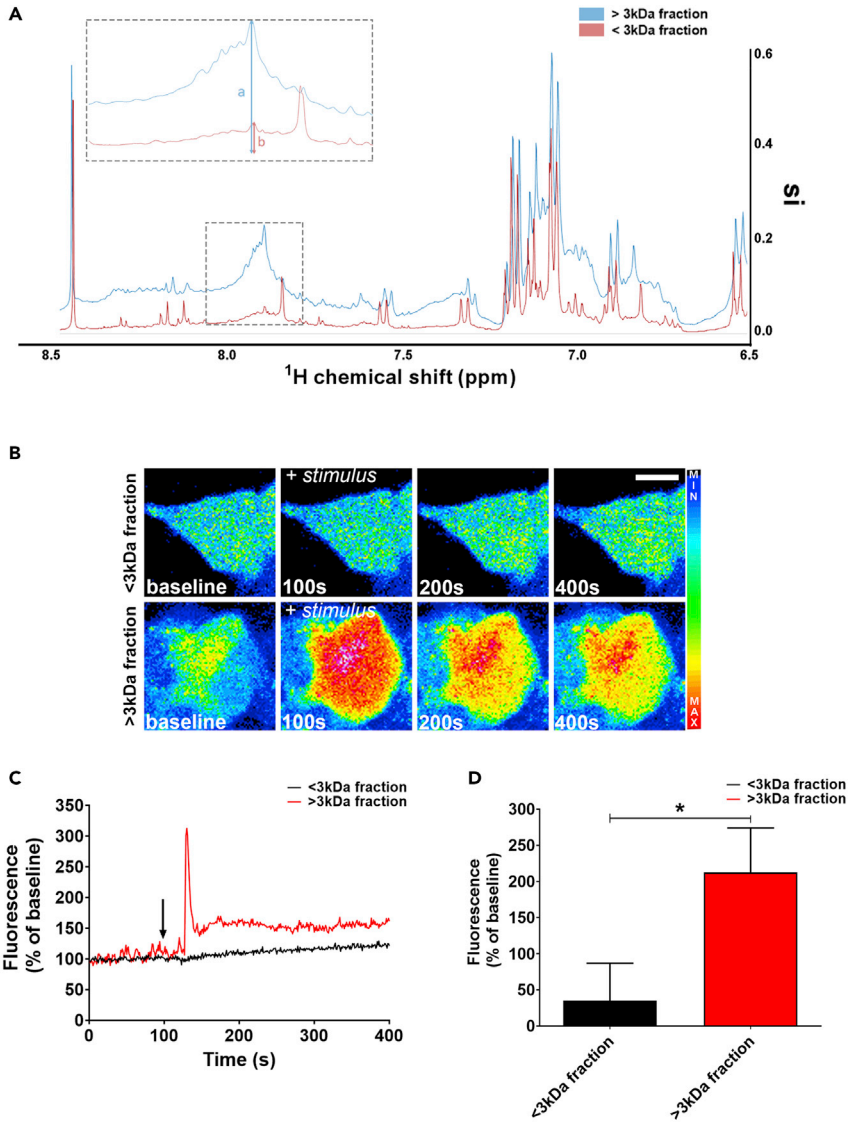

Figure 1. *Akkermansia muciniphila* conditioned medium characterization and experimental application

(A) Protein side-chain H^N NMR spectrum evaluated by 500 MHz ^1H NMR spectra of > 3 kDa fraction of *Akkermansia muciniphila* conditioned BHI (black line) and BHI+0.4% mucin (red line) and < 3 kDa fraction (flow-through) of BHI CM (blue line).

(B) Confocal microscopy imaging of STC-1 cells incubated with Fluo-4/AM (6 μM) and stimulated with 10% (v/v) >3 kDa and <3 kDa fraction of *A. muciniphila* conditioned medium (BHI CM) (scale bar: 10 μm) Images were pseudocolored for fluorescence intensity according to the scale shown on the right.

(C) Representative time-course of total Ca^{2+} signal. Arrow indicates the moment when culture medium was applied.

(D) Quantification of the peak fluorescence following stimulation with 10% (v/v) >3 kDa and <3 kDa fraction of *A. muciniphila* conditioned medium (BHI CM). Error bars indicate the media ± SD n= at least 25 cells for each group, * p< 0.05 by unpaired Student's t-test.

a. Properly identify the centricon cover and body.
b. Keep the original tubes containing BHI CM and BHI in an ice bath during the entire procedure.

12. Place the centricons in the centrifuge so that the weight is balanced inside the centrifuge and centrifuge at 5,000 g, 4°C for 20 min.
13. Then, using a micropipette, make repeated flush in the center of the centricon membrane to homogenize the fraction being concentrated. Be careful not to touch the membrane with the tip, to prevent tearing.

14. Repeat steps 11–13 as many times as necessary until the BHI CM and BHI are concentrated in the top of the centricon to a 3 mL volume.

Note: The <3 kDa fraction (retained in the lower portion of the centricon) will probably increase in volume rapidly in the centricon. Do not let it reach the membrane as this will hinder the concentration process. If that happens, transfer this volume to a new sterile 50 mL Falcon tube, properly identified. Keep it in on ice (4°C).

⚠ CRITICAL: Be careful to not mix >3 kDa and <3 kDa fractions, or BHI CM with BHI. Always dispose used micropipette tips. Always identify the cap and body of the tubes and centricon used.

⚠ CRITICAL: To avoid thawing cycles, it is recommended that this entire step of fractionation the BHI CM and BHI be done on the same day.

15. Once fractionated, collect the BHI CM and BHI >3 kDa and <3 kDa fractions in properly identified sterile 15 mL tubes.
16. Then, with the help of a syringe attached to a 0.22 μm filter, sterilize each of the fractions by transferring them to new sterile and identified containers.
17. Aliquot and immediately store them at −80°C until the moment of use.

Note: The fractions can be stored in sterile microtubes of 3 mL (or less) or divided into smaller portions containing volumes suitable for use. This form of storage is interesting because it avoids thawing cycles of the samples.

Intracellular calcium signaling imaging

Timing: 4–5 h

This step describes the enteroendocrine cell line STC-1 cultivation, and how to stimulate them using the *Akkermansia muciniphila* conditioned medium fractions in an intracellular Ca^{2+} Signaling experiment (Figures 1B and 1C).

18. Once the desired confluence is reached (70%–80% is ideal), dilute the desired Ca^{2+} indicator (in our example, Fluo-4/AM) according to the manufacturer's specifications (see at Fluo-4/ AM calcium indicator) and keep it in an ice bath. Transfection of cells with Ca^{2+} indicator plasmids (e.g., GCaMP) can also be performed.
19. Turn on the CO_2 and heating of the confocal microscope stage by setting it to 37°C. Adjust the laser settings and parameters for live imaging.
    a. The experiments in this protocol were performed on a Leica SP8 confocal microscope using a ×63 objective lens, 1.4 NA.
    b. Excitation at 488 nm and emission at 505–525 nm.
    c. Frame scanning module at a 15 f/s during 5 min of imaging is an ideal parameter.

Note: Other parameters such as zoom or laser intensity, for example, may vary in microscopes at different locations, and therefore should be standardized in advance by the executors of this protocol.

20. Add the fluorescent probe for cytosolic calcium detection Fluo-4/AM to one of the wells of the STC-1 plate, according to the concentration indicated by the probe manufacturer (see at Fluo-4/ AM calcium indicator), and keep it at 37°C for 15–20 min.

21. Then remove from the 6-well plate one of the glass coverslips on which the STC-1 cells have grown adhered and transfer it to an imaging chamber on a stage of a Leica SP8 Confocal System and continuously perfused with HEPES buffer solution.
22. Select an image field where the cells are spread out and the initial fluorescence emission is homogeneous.
23. Start live-image recording of the cell in its basal state for approximately 100 s to acquire a baseline of fluorescence. Then add the desired stimulus, for example BHI CM (>3 kDa) 1 or 10% (v/v) and keep recording the response for approximately another 300 s.
    a. Save and properly name the obtained image file in a folder of the repository where your lab's confocal microscopy data is located.
24. Repeat steps 15–18 using another well of the STC-1 plate, and this time stimulate the cells with 10% (v/v) BHI (>3 kDa) as the experiment negative control.

Note: For each variable (for example, <3 and >3 kDa BHI CM and BHI fractions at different concentrations of 1 and 10% (v/v)) an individual experiment should be performed at least 3 times. Around 25 cells per coverslip/chamber are analyzed.

Optional: Other experimental approaches can be performed by adapting this protocol. By incubating the cells in Ca^{2+}-free HEPES buffer, one can obtain information on whether or not the intracellular Ca^{2+} signaling observed is dependent on extracellular Ca^{2+} stocks.

25. Data are expressed as fluorescence/baseline fluorescence × 100% of the average values of samples from at least 3–6 biological replicates (≥25 cells/replicate).
    a. This type of experimental approach allows to obtain a representative figure (or video) of the cells in their basal and stimulated condition and a graph of the Ca^{2+} response profile to the stimulus.
    b. It is also possible to quantify the maximum and mean fluorescence amplitude obtained upon stimulation, and they are related to the increased Ca^{2+} fluctuation in the cells.
    c. See Figure 1.

EXPECTED OUTCOMES

Ca^{2+} signaling is one of the pathways by which the cell maintains its homeostasis. Disturbances in Ca^{2+} concentrations in the cell are directly associated with Parkinson's Disease pathogenesis and αSyn aggregation (Guzman et al., 2010; Lowe et al., 2009; Nath et al., 2011; Surmeier, 2007) and for this reason this protocol may be useful for screening the action of *Akkermansia* but also other microorganisms in EECs in the Parkinson's Disease context.

LIMITATIONS

This protocol was written with a focus on *Akkermansia muciniphila* CM. Originally the *Escherichia coli* CM was also fractionated and also concentrated into >3 kDa and <3 kDa fractions and used to stimulate STC-1 cells in different experimental approaches (Amorim Neto et al., 2022). However, NMR analysis of the concentrated fractions was done only for BHI CM from *Akkermansia muciniphila*. Although this protocol can be applied to other microorganisms, the distinction of protein- and metabolite-rich fractions must be confirmed in further studies.

TROUBLESHOOTING

Problem 1

Excessive probe internalization can lead to cytotoxicity (step 20).

Potential solution

Plan the timing of loading each of the STC-1 wells so that the cells do not exceed the incubation time with the probe (15–20 min).

Problem 2

High-intensity laser may induce cell death or cell injury (step 19).

Potential solution

Standardize the confocal microscope setup to use the laser at the lowest possible power, but still allow excitation and capture of the fluorescence emitted by the probe.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Matheus Fonseca (mdecastr@caltech.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.C.F. and D.P.A.N.; Formal Analysis, D.P.A.N. and M.C.F.; Investigation, D.P.A.N.; Resources, M.C.F.; Writing, D.P.A.N. and M.C.F.; Funding Acquisition, M.C.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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