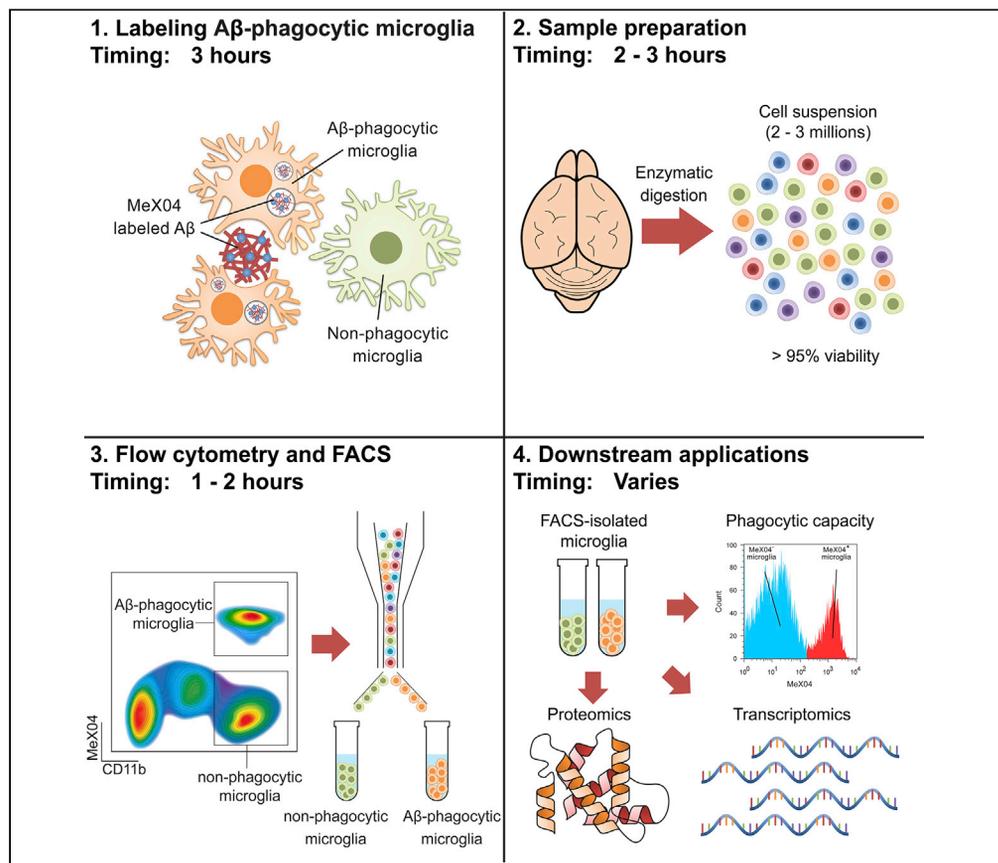


## Protocol

# Quantitative *in vivo* assessment of amyloid-beta phagocytic capacity in an Alzheimer's disease mouse model



Alzheimer's disease is characterized by the deposition of extracellular amyloid-beta (A $\beta$ ) plaques. While microglial phagocytosis is a major mechanism through which A $\beta$  is cleared, there is no method for quantitatively assessing A $\beta$  phagocytic capacity of microglia *in vivo*. Here, we present a flow cytometry-based method for investigating the A $\beta$  phagocytic capacity of microglia *in vivo*. This method enables the direct comparison of A $\beta$  phagocytic capacity between different microglial subpopulations as well as the direct isolation of A $\beta$  phagocytic microglia for downstream applications.

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### HIGHLIGHTS

Detailed protocol for quantitative assessment of A $\beta$  phagocytosis *in vivo*

Optimized sample preparation for isolating abundant and highly viable microglia

High-throughput analysis of microglial phagocytic capacity within 1 day

Compatible with downstream transcriptomic and proteomic analyses

Lau et al., STAR Protocols 2, 100265

March 19, 2021 © 2020 The Authors.

<https://doi.org/10.1016/j.xpro.2020.100265>



## Protocol

Quantitative *in vivo* assessment of amyloid-beta phagocytic capacity in an Alzheimer's disease mouse modelShun-Fat Lau,<sup>1,2,4</sup> Wei Wu,<sup>1,2</sup> Heukjin Seo,<sup>1,2</sup> Amy K.Y. Fu,<sup>1,2,3</sup> and Nancy Y. Ip<sup>1,2,3,5,\*</sup><sup>1</sup>Division of Life Science, State Key Laboratory of Molecular Neuroscience, Molecular Neuroscience Center, The Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China<sup>2</sup>Hong Kong Center for Neurodegenerative Diseases, Hong Kong, China<sup>3</sup>Guangdong Provincial Key Laboratory of Brain Science, Disease and Drug Development, HKUST Shenzhen Research Institute, Shenzhen-Hong Kong Institute of Brain Science, Shenzhen, Guangdong 518057, China<sup>4</sup>Technical contact<sup>5</sup>Lead contact\*Correspondence: [boip@ust.hk](mailto:boip@ust.hk)<https://doi.org/10.1016/j.xpro.2020.100265>

## SUMMARY

Alzheimer's disease is characterized by the deposition of extracellular amyloid-beta (A $\beta$ ) plaques. While microglial phagocytosis is a major mechanism through which A $\beta$  is cleared, there is no method for quantitatively assessing A $\beta$  phagocytic capacity of microglia *in vivo*. Here, we present a flow cytometry-based method for investigating the A $\beta$  phagocytic capacity of microglia *in vivo*. This method enables the direct comparison of A $\beta$  phagocytic capacity between different microglial subpopulations as well as the direct isolation of A $\beta$  phagocytic microglia for downstream applications.

For complete details on the use and execution of this protocol, please refer to Lau et al. (2020).

## BEFORE YOU BEGIN

⌚ Timing: 1–1.5 h

This section lists the stock and working solutions needed for the experiment. It is strongly recommended to prepare the working solution immediately before the experiments.

1. Prepare methoxy-X04 (MeX04) solution
  - a. Stock solution (10 mg/mL): Prepare MeX04 stock solution (10 mg/mL) by dissolving in DMSO and storing in aliquots (200  $\mu$ L) at  $-80^{\circ}\text{C}$  for at least 1 year.
  - b. Working solution (2 mg/mL): Dilute 2 parts MeX04 stock in 3 parts DMSO and 5 parts 0.9% saline (pH 12.0). Refer to "Materials and equipment" for detailed recipe for dilution.
2. Prepare artificial cerebrospinal fluid (aCSF) for brain dissociation
  - a. aCSF stock solution (10 $\times$ , without bicarbonate and glucose): Refer to "Materials and equipment" for detailed recipe.
  - b. aCSF working solution (1 $\times$ ): Refer to "Materials and equipment" for detailed recipe for dilution.
3. Enzyme dissociation solution (20 mL per reaction, freshly prepared)
  - a. Refer to "Materials and equipment" for detailed recipe.
4. Isotonic Percoll (stock at  $4^{\circ}\text{C}$ )
  - a. Refer to "Materials and equipment" for detailed recipe.



## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Rat anti-CD11b (M1/70) APC	eBioscience	Cat. #17-0112-83
Rat anti-CD45 (30-F11) FITC	eBioscience	Cat. #11-0451-85
<b>Chemicals, peptides, and recombinant proteins</b>		
Methoxy-X04	Tocris Bioscience	Cat. #4920
Papain	Worthington Biochemical	Cat. #LS003126
DNaseI	Worthington Biochemical	Cat. #LS002140
L-Cysteine	Sigma-Aldrich	Cat. #168149
Percoll	Sigma-Aldrich	Cat. #P1644
HBSS (10×), no calcium, no magnesium, no phenol red	Thermo Fisher	Cat. #14185052
DMEM/F-12, no phenol red	Thermo Fisher	Cat. #21041025
Fetal bovine serum	Corning	Cat. #35-079-CV
Dimethyl sulfoxide	Sigma-Aldrich	Cat. #472301
<b>Experimental models: organisms/strains</b>		
APP/PS1 mice (non-transgenic mice as WT mice control)	The Jackson Laboratory	Cat. #34829-JAX
<b>Software and algorithms</b>		
FlowJo	TreeStar	<a href="https://www.flowjo.com">https://www.flowjo.com</a>
<b>Other</b>		
Hemocytometer	Sigma	Cat. #Z359629
BD Influx Cell Sorter	BD Biosciences	n/a

## MATERIALS AND EQUIPMENT

### MeX04 working solution (for 3 injections, freshly prepared)

Reagent	Final concentration	Volume
MeX04 stock (10 mg/mL)	2 mg/mL	200 $\mu$ L
DMSO	n/a	300 $\mu$ L
0.9% saline, pH 12.0	n/a	500 $\mu$ L
<b>Total</b>	n/a	1,000 $\mu$ L

△ **CRITICAL:** Protect from light. Avoid repeated freeze-thaw of MeX04 stock. The resultant MeX04 working solution (2 mg/mL) should be yellow.

### aCSF stock solution (10×, stock at 4°C for at least 1 month)

Reagent	Final concentration	Amount or volume
NaCl	1190 mM	34.77 g
KCl	25 mM	0.93 g
CaCl <sub>2</sub> ·2H <sub>2</sub> O	25 mM	1.84 g
NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	10 mM	0.78 g
MgCl <sub>2</sub> ·6H <sub>2</sub> O	13 mM	1.32 g
Autoclaved deionized H <sub>2</sub> O	n/a	To 500 mL
<b>Total</b>	n/a	500 mL

### aCSF working solution (1×, freshly prepared)

Reagent	Final concentration	Amount or volume
aCSF stock solution	1 ×	50 mL
NaHCO <sub>3</sub>	26.2 mM	1.1 g
D-Glucose	11.0 mM	0.991 g
Autoclaved deionized H <sub>2</sub> O	n/a	To 500 mL
<b>Total</b>	n/a	500 mL

Enzyme dissociation solution (20 mL per reaction, freshly prepared)		
Reagent	Final concentration	Volume
aCSF working solution	1 ×	19.8 mL
Papain (1,000 U/mL)	5 U/mL	100 μL
DNaseI (7,000 U/mL)	35 U/mL	100 μL
L-Cysteine	1.65 mM	0.004 g
<b>Total</b>	<b>N/A</b>	<b>20 mL</b>

Isotonic Percoll (stock at 4°C for at least 3 months)		
Reagent	Final concentration	Volume
10× HBSS	1 ×	5 mL
Percoll	n/a	45 mL
<b>Total</b>	<b>n/a</b>	<b>50 mL</b>

## STEP-BY-STEP METHOD DETAILS

### *In vivo* labeling of amyloid plaques

⌚ Timing: 3 h

This section describes how to label amyloid plaques with methoxy-X04 (MeX04), a blood–brain barrier-penetrating amyloid-beta (Aβ) dye. After MeX04 is intraperitoneally injected into Alzheimer’s disease (AD) transgenic mice, it enters the brain and binds to the surface of Aβ plaques. If microglia are actively phagocytosing amyloid plaques, MeX04 will be internalized together with Aβ. Thus, subsequent flow cytometry analysis enables the quantification of the capacity of microglia to phagocytose Aβ within the 3 h interval between MeX04 injection and sacrifice.

1. Intraperitoneal injection of MeX04 into 6–18-month-old AD transgenic mice
  - a. Warm the MeX04 working solution (2 mg/mL) in a 37°C water bath for 5 min before injection.
  - b. Weigh the AD transgenic mice and intraperitoneally inject MeX04 working solution at 10 mg/kg.

**Note:** Also include non-transgenic wild-type (WT) mice (i.e., without amyloid plaques) to control for MeX04 signal baseline in flow cytometry analysis.

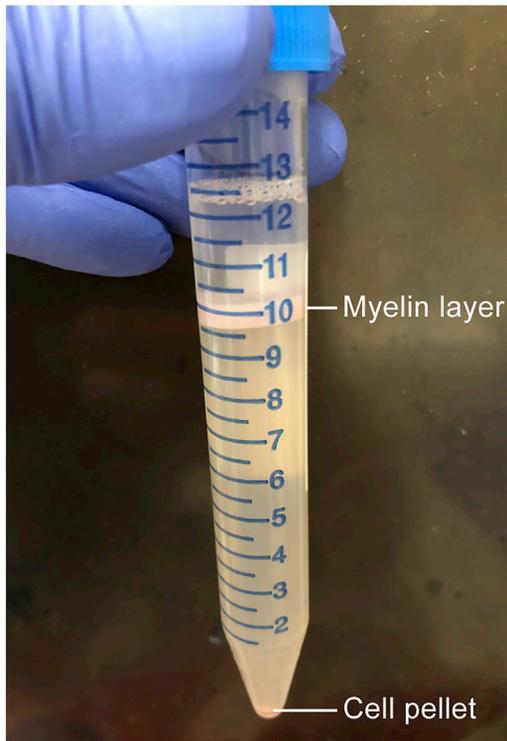
### Brain dissociation and microglia isolation

⌚ Timing: 3–4 h

This section describes a modified protocol to maximize the number of microglia isolated.

⚠ **CRITICAL:** To achieve a high yield of viable microglia, freshly prepared aCSF with pH 7.2–7.4 and gentle mechanical dissociation are essential. It is also recommended that each experimenter handle fewer than 3 samples to minimize differences in digestion time.

2. Brain dissection
  - a. Euthanize mice using isoflurane 3 h after MeX04 injection and perfuse mice with 20 mL ice-cold PBS.
  - b. Dissect the region(s) of interest in ice-cold PBS.
3. Brain dissociation by papain
  - a. Prepare enzyme dissociation solution by adding 0.004 g L-cysteine, 100 U papain, and 700 U DNaseI into 20 mL working aCSF solution (refer to “[Materials and equipment](#)” for detailed recipe).

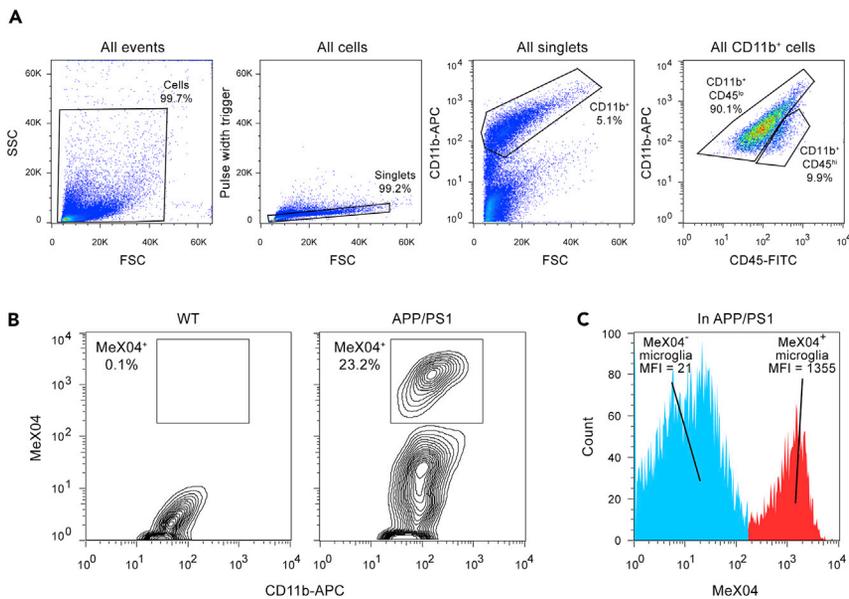


**Figure 1. Photograph of the myelin layer and cell pellet after Percoll gradient centrifugation**

The myelin layer is trapped between the 30% Percoll layer and artificial cerebrospinal fluid layer.

- b. Prewarm the enzyme dissociation solution in a 37°C water bath for 5 min.
- c. Mince the brain tissue into small pieces on a petri dish and transfer to a 50-mL falcon tube with 20 mL enzyme dissociation solution.
- d. Incubate in a 37°C water bath with constant shaking for 10 min.
- e. Remove the tube from the water bath and let the undigested tissue sink to the bottom.
- f. Very gently pass the tissue through a 20-gauge needles 3 times and return the tubes to the 37°C water bath for an additional 30 min of digestion.
- g. Pellet the cells by centrifugation at 800 × g at 4°C for 5 min.
- h. Aspirate the supernatant and subject it to a Percoll gradient.
4. Percoll gradient
  - a. Prepare isotonic Percoll by adding 9 parts Percoll into 1 part 10× HBSS.
  - b. Resuspend the cell pellet in 7 mL working aCSF solution.
  - c. Add 3 mL isotonic Percoll and mix well by inverting 10 times; the resultant solution contains 30% Percoll.
  - d. Gently add 2 mL working aCSF on top.
  - e. Centrifuge at 800 × g at 4°C for 15 min with the slowest acceleration and zero deceleration.
  - f. Use a P1000 pipette to gently remove the myelin debris layer trapped between the aCSF and 30% Percoll, and subsequently aspirate all supernatant (Figure 1).
  - g. Resuspend the cell pellet in 200 μL of ice-cold DMEM/F12 with 10% heat-inactivated FBS.
5. Cell staining
  - a. Before cell staining, aliquot 5 μL cell suspension and inspect cell viability, number, and singlet by hemocytometer.

**Note:** Number of viable cell number ranges from 1.5–3.0 × 10<sup>6</sup> per forebrain depending on age of AD transgenic mice.



**Figure 2. Gating strategy and analysis of A $\beta$  phagocytic microglia in APP/PS1 transgenic mice**

(A) Gating strategy to identify CD11b<sup>+</sup> CD45<sup>lo</sup> microglia. SSC, side-scattered.

(B) Contour plots showing the proportion of A $\beta$  phagocytic microglia in 16-month-old APP/PS1 mice (an Alzheimer’s disease transgenic mouse model). Age-matched non-transgenic wild-type (WT) mice, which lack MeX04 signal, are used as control.

(C) Histogram showing the MeX04 signal intensity of A $\beta$  phagocytic microglia (i.e., MeX04<sup>+</sup> microglia) and non-A $\beta$  phagocytic microglia (i.e., MeX04<sup>-</sup> microglia). MFI, mean fluorescence intensity.

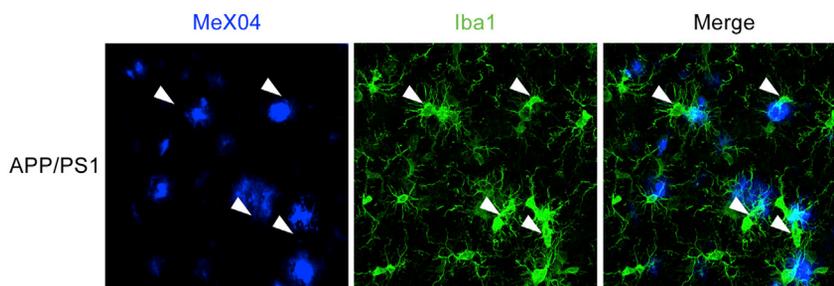
- b. To label microglia, stain the cell suspension (200  $\mu$ L) with CD11b-APC (1:200) and CD45-FITC (1:200) for 45 min at 4°C with rotation. In addition, prepare unstained controls using AD transgenic model mice and non-transgenic wild-type mice to serve as fluorescence-minus-one controls.

**Note:** The emission spectrum of MeX04 overlaps with that of DAPI/Pacific blue.

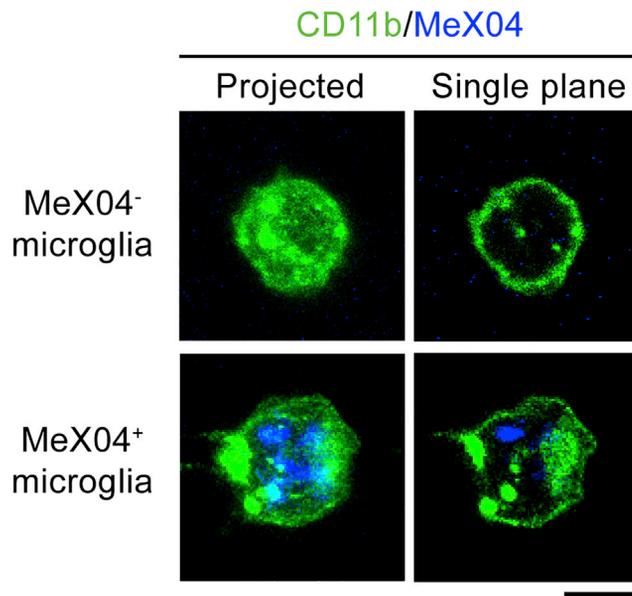
- c. After 45 min, wash the cells with 1 mL DMEM/F12 + 10% FBS and centrifuge at 800  $\times$  g at 4°C for 5 min.
- d. Resuspend the cells in 500  $\mu$ L DMEM/F12 + 10% FBS.

## 6. Flow cytometry

- a. Switch on the flow cytometer and perform machine start-up.
- b. Create the following plots in the template (Figures 2A and 2B)
  - i. Side-scattered (SSC) versus forward-scattered (FSC) (to identify cell populations)
  - ii. Pulse width trigger versus FSC (to identify singlets)



**Figure 3. Representative images showing the proportions of A $\beta$ <sup>+</sup> microglia in 16-month-old APP/PS1 mice**  
Scale bar, 20  $\mu$ m.



**Figure 4. Intracellular MeX04 in FACS-isolated MeX04<sup>+</sup> microglia**  
Scale bar, 5 μm.

- iii. PB488 versus FSC-A (to identify CD45 signal)
- iv. PB660 versus FSC-A (to identify CD11b signal)
- v. PB450 versus PB660 (to identify MeX04 signal)
- c. Use a non-transgenic mice brain sample to set a negative signal for MeX04 and an unstained control sample to set a negative signal for CD11b and CD45.
- d. Proceed with the samples and record the MeX04 signals of 10,000 microglia.
- e. Analyze the data using FlowJo to obtain the following:
  - i. Proportion of MeX04<sup>+</sup> microglia = proportion of Aβ phagocytic microglia (Figure 2B)
  - ii. Mean fluorescence intensity of MeX04 = Aβ phagocytic capacity (Figure 2C)

## EXPECTED OUTCOMES

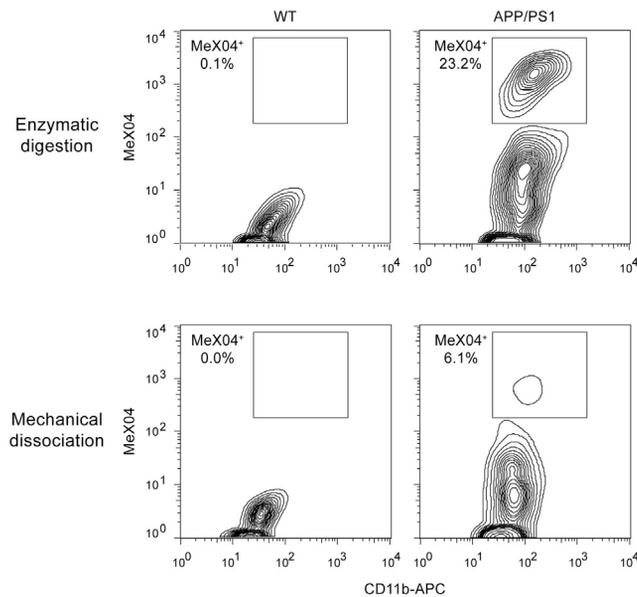
This section describes some of the quality control steps during the protocol and the anticipated results. These steps are recommended to ensure good-quality results and minimize artifacts due to sample preparation.

### Cell number and viability after papain dissociation

When starting with the whole forebrain, 2–3 million viable cells are routinely obtained after sample preparation. More importantly, the cell suspension should be mostly viable (>95%) and singlet. After FACS isolation, approximately 150,000 microglia per 12-month-old APP/PS1 mouse are usually obtained; more if a younger mouse is used.

**Table 1. Advantages and disadvantages of using enzymatic and mechanical dissociation in this protocol**

Characteristic	Enzymatic dissociation	Mechanical dissociation
Cell viability	Higher	Lower
Proportion of Aβ phagocytic microglia	Higher (due to better cell viability; Figure 5)	Lower (due to poor cell viability; Figure 5)
Preservation of transcriptome	Potential induction of immediate early genes	Better



**Figure 5. Representative contour plots showing the proportions of A $\beta$  phagocytic microglia after enzymatic or mechanical digestion**

WT, non-transgenic wild-type.

### Proportion of A $\beta$ phagocytic microglia

The proportion of A $\beta$  phagocytic microglia varies among AD transgenic mouse models (e.g., APP/PS1 and 5xFAD mice) and with respect to mouse age. For APP/PS1 mice, the proportion of A $\beta$  phagocytic microglia should be  $\sim$ 10% and  $\sim$ 20% at 12 and 16 months of age, respectively (Figure 2B) (Fu et al., 2016; Lau et al., 2020). This result has been further validated by immunohistochemistry (IHC) in the same APP/PS1 mice. Flow cytometry analysis showed that in 16-month-old APP/PS1 mice,  $20.4 \pm 2.8\%$  of microglia are MeX04+ ( $n = 4$ ; Figure 2B), whereas IHC analysis showed that  $27.9 \pm 4.0\%$  of microglia are A $\beta$ + ( $n = 4$ ; Figure 3). These results demonstrate the consistency of our method for examining the proportion of A $\beta$  phagocytic microglia. Also, the intracellular localization of MeX04 in A $\beta$  phagocytic microglia can be confirmed by confocal imaging (Figure 4).

### LIMITATIONS

This protocol only measures the phagocytic capacity of microglia within 1–2 h of MeX04 injection. It is inappropriate for long-term measurement (i.e., more than 4 h), because MeX04 can nonspecifically bind to nuclei or other non-A $\beta$  materials.

Although this protocol is compatible with mechanical dissociation, there will be substantially more dead cells as a result of mechanical dissociation. The advantages and disadvantages of enzymatic dissociation versus mechanical dissociation are summarized below (Table 1).

### TROUBLESHOOTING

#### Problem 1

The MeX04 working solution appears cloudy (step 1a).

#### Potential solutions

Ensure that the 0.9% saline has the correct pH of 12.0.

Use new anhydrous DMSO.

Use a new MeX04 stock solution.

### Problem 2

MeX04 cross-reacts with non-A $\beta$  materials (step 1b).

### Potential solutions

Titrate MeX04 for intraperitoneal injection and examine the specificity of the A $\beta$  staining pattern by co-staining with A $\beta$  antibody on brain sections.

### Problem 3

There are few cells and/or poor viability after sample preparation (step 5a).

### Potential solutions

Freshly prepare all buffers and bubble 5% CO<sub>2</sub> in O<sub>2</sub> through them for at least 10 min immediately before the experiment.

Gently mechanically dissociate with a 20-gauge needle; to avoid foaming, eject toward the tube wall.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Nancy Y. Ip (e-mail: [boip@ust.hk](mailto:boip@ust.hk)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate any unique datasets or code.

## ACKNOWLEDGMENTS

We thank all members of the Ip Laboratory for their many helpful discussions. This study was supported in part by the Research Grants Council of Hong Kong (the Collaborative Research Fund [C6027-19GF], the Theme-Based Research Scheme [T13-607/12R], and the General Research Fund [HKUST16149616, HKUST16103017, HKUST16100418, and HKUST16102019]), the National Key R&D Program of China (2017YFE0190000 and 2018YFE0203600), the Areas of Excellence Scheme of the University Grants Committee (AoE/M-604/16), the Innovation and Technology Commission (ITCPD/17-9), the Guangdong Provincial Key S&T Program (2018B030336001), and the Shenzhen Knowledge Innovation Program (JCYJ20180507183642005 and JCYJ20170413173717055). S.-F.L., W.W., and H.S. are recipients of the Hong Kong PhD Fellowship Award.

## AUTHOR CONTRIBUTIONS

S.-F.L., A.K.Y.F., and N.Y.I. conceived of the project; S.-F.L., W.W., and H.S. optimized and conducted the experiments; and S.-F.L., A.K.Y.F., and N.Y.I. wrote the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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