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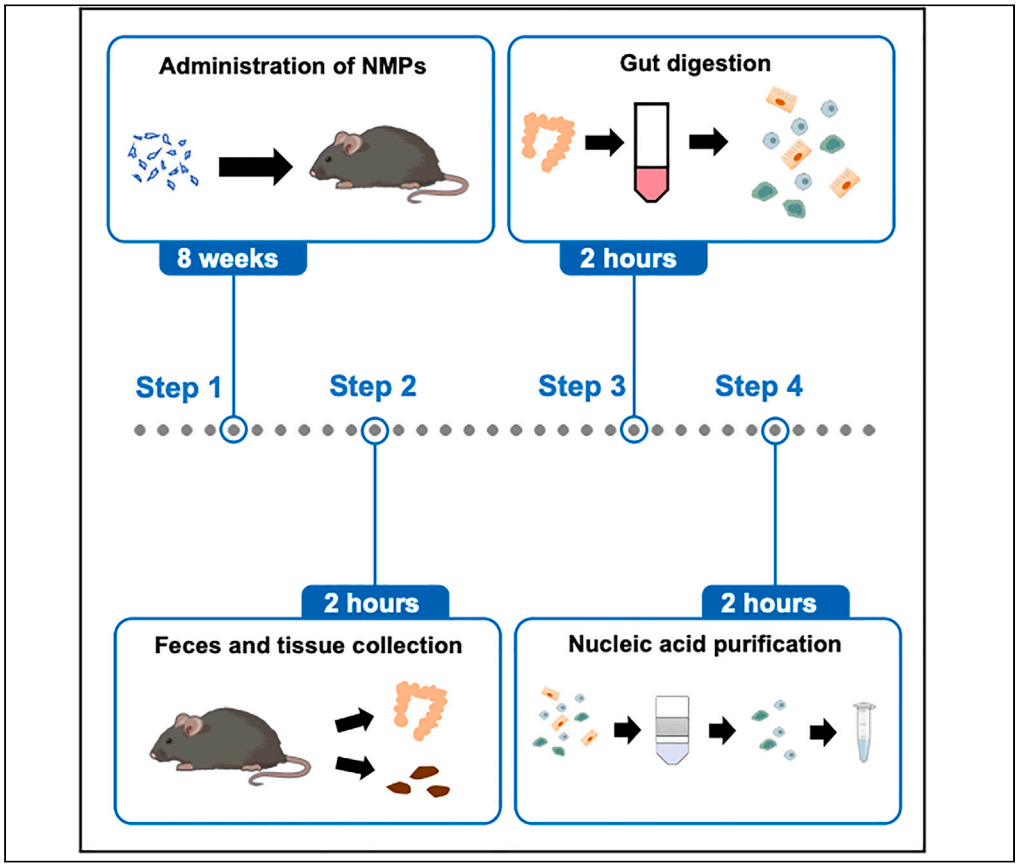
Protocol for acquiring samples to assess the impact of microplastics on immune microenvironments in the mouse intestine



Environmental nano- and microplastics (NMPs) pose serious environmental issues, yet there is no established technique to assess their impact on health through oral ingestion. Here, we present a protocol to assess the impact of NMPs in the intestinal immune microenvironments by employing chronic exposure to NMPs in a mouse model. We describe steps for administration of NMPs, feces and tissue collection, and colonic gut digestion. We then detail procedures for isolation of intestinal immune cells and RNA isolation.

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

Akihito Harusato, Wooseok Seo, Hirohito Abo, Yoshitaka Nakanishi, Hiroyoshi Nishikawa, Yoshito Itoh

harup@koto.kpu-m.ac.jp

Highlights

Protocol to assess the impact of ingested microplastics

Optimized protocol for low-dose NMP administration in mice

Detailed steps for collecting samples for transcriptome and microbiome analysis

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Protocol for acquiring samples to assess the impact of microplastics on immune microenvironments in the mouse intestine

Akihito Harusato,[1,5,6,*] Wooseok Seo,[2] Hirohito Abo,[3] Yoshitaka Nakanishi,[4] Hiroyoshi Nishikawa,[2] and Yoshito Itoh[1]

[1]Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan

[2]Department of Immunology, Nagoya University Graduate School of Medicine, Nagoya, Japan

[3]Institute for Biomedical Sciences, Georgia State University, Atlanta, GA, USA

[4]Faculty of Advanced Science and Technology, Kumamoto University, Kumamoto, Japan

[5]Technical contact

[6]Lead contact

*Correspondence: harup@koto.kpu-m.ac.jp
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SUMMARY

Environmental nano- and microplastics (NMPs) pose serious environmental issues, yet there is no established technique to assess their impact on health through oral ingestion. Here, we present a protocol to assess the impact of NMPs in the intestinal immune microenvironments by employing chronic exposure to NMPs in a mouse model. We describe steps for administration of NMPs, feces and tissue collection, and colonic gut digestion. We then detail procedures for isolation of intestinal immune cells and RNA isolation.

For complete details on the use and execution of this protocol, please refer to Harusato et al.[1]

BEFORE YOU BEGIN

This protocol will demonstrate *in vivo* chronic exposure model of particulate microplastics (<5 μm in size) and will acquire fecal samples and isolated intestinal immune cells. This protocol has been modified based on previous works to efficiently isolate intestinal immune cells for functional analysis.[2–5]

> *Optional:* This protocol describes a protocol for isolating colonic lamina propria immune cells. However, we have also utilized this protocol to isolate small intestinal lamina propria immune cells with minor modifications (See also Geem D et al.[3] and Harusato A et al.[4]).

Institutional permissions for animal experiment

All experiments were implemented in accordance with procedures approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University of Medicine, Kyoto, Japan. All mice were kept at 18°C–24°C with 40%–70% humidity and maintained under a 12 h/12 h dark/light cycle.

Mice

Mucosal immune responses in the gut are not only affected by the composition of gut microbiota,[6] but also by several elements including vivarium, age, sex and strain.[7–9] Thus, all of these elements need to be matched in each experimental group before starting the experiments. Hence, mice

were obtained from the same vendor for all the experiments. All of wild-type 6 weeks old C57BL/6J female mice were purchased from the Shimizu Laboratory Supplies Co. Ltd (Japan).

Optional: Previous studies demonstrated that co-housing and bedding transfers are effective ways to have reproducible results of microbiota analysis.[10,11] In cases where the number of mice is limited, a co-housing approach should be considered. In cases where using a large number of mice, bedding transfers are a more effective approach.

Administration of NMPs

Timing: 8 weeks

1. Prepare nano- and microplastics (NMPs) subject to investigation. As for the manufacturing process of NMPs, refer to Nakanishi et al.[12,13]
2. Suspend NMP into a Ca^{2+}/Mg^{2+}-free PBS (CMF PBS) in a 15 mL tube. Mix NMPs by Vortex 20 s. Make aliquots in 1.5 mL tubes, which makes it easier to suck up the contents in Step 4.
3. To avoid the loss of NMPs, use an ultrasonic water bath for 5 min to detach NMPs from the internal surfaces of the 15 mL tube or 1.5 mL tube.
4. Attach the intragastric feeding needle at the tip of a 1 mL syringe. After a short Vortex, fill NMP-containing CMF PBS into a 1 mL syringe.
5. Orally deliver 100 μL of CMF PBS containing 3×10^4 NMPs to each mouse. Oral administration was repeated once every three days for 8 weeks.

Alternatives: The dose, frequency and duration of NMP oral administration can be modified depending on the experimental purpose. As for the sources of NMP, see also Nakanishi Y et al.[12,13] Alternatively, various sizes of NMPs derived from several plastic resins are commercially available.

⚠ CRITICAL: To obtain environmentally relevant results, NMPs should not be administered in excess as several previous *in vivo* studies employed extraordinarily high doses of polystyrene spherical beads.[14,15] According to Oßmann et al.,[16] the dose employed in this protocol (3×10^4 NMPs/3 days) corresponds with the consumption of 2–3 PET bottles per day in real life.

Preparation of reagents and equipment

Timing: 0.5 h

6. Prepare buffers and reagents below:
   a. Warm CMF PBS to room temperature.
   b. Prepare ice-cold Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS) with 5% of Fetal Bovine Serum (FBS) (CMF HBSS/FBS).
   c. Warm CMF HBSS/FBS with 2 mM EDTA to 37°C to wash off epithelium.
   d. Prepare collagenase solution: 1.5 mg/mL Type IV Collagenase dissolved in CMF HBSS/FBS with 40 μg/mL of DNase I at 37°C.
7. Pre-heat orbital shaker to 37°C.

Washing buffer

Reagent	Stock concentration	Final concentration	Amount
Ca^{2+}/Mg^{2+}-free HBSS	1×	1×	94.8 mL
FBS	N/A	5% (v/v)	5 mL
EDTA	1 M	2 mM	200 μL
Total			**100 mL**

Collagenase solution			
Reagent	Stock concentration	Final concentration	Amount
Ca^{2+}/Mg^{2+}-free HBSS	1×	1×	94,8 mL
FBS	N/A	5% (v/v)	5 mL
Type IV Collagenase	N/A	1.5 mg/mL	150 mg
DNase I	20mg/ml	40μg/ml	200 μl
Total			**100 mL**

⚠ CRITICAL: The prepared buffer should be used on the day of experiments and cannot be stored. The concentration of collagenase can be modified. Since the collagenase activity may vary in each lot number, collagenase should be tested before using a new lot of collagenase. Optimizing the concentration of collagenase as well as the duration of tissue digestion is required to generate a vigorous cell yield without affecting cell viability. As a side note, cell viability can be checked by Live/Dead staining by flow cytometry.[3]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
HBSS buffer	Fujifilm	#084-08345
PBS	Fujifilm	#166-23555
MACS BSA stock solution	Miltenyi	#130-091-376
autoMACS rinsing solution	Miltenyi	#130-091-222
FBS	N/A	N/A
EDTA	N/A	N/A
Collagenase	Sigma-Aldrich	C5138
DNase I	Roche	#10104159001
Ertalyte PET-P	Mitsubishi Chemical	N/A
Critical commercial assays		
RNeasy Plus Micro Kit (including RLT buffer)	QIAGEN	#74034
CD45 MicroBeads, mouse	Miltenyi	#130-052-301
Experimental models: Organisms/strains		
Mouse: C57BL/6J (*Mus musculus*, 6-week-old, female)	Shimizu Laboratory Supplies	N/A
Other		
100 μm cell strainer	BD Falcon	#352350
40 μm cell strainer	BD Falcon	#352340
Feeding needles	Fuchigami	#6202
1.5 mL tubes	N/A	N/A
15 mL tubes	N/A	N/A
MACS MultiStand	Miltenyi	#130-042-303
MidiMACS separator	Miltenyi	#130-042-301
LS columns	Miltenyi	#130-042-401
NanoDrop 2000	Thermo Scientific	N/A
Orbital shaker	WakenBtech	StackShakeC
Centrifuge	TOMY	AX-310
Vortex	Scientific Industries	Vortex-Genie 2
Ultrasonic washer	AS ONE	USM-1
Surgical scissors and forceps	Natsume	N/A
1 mL syringe	Terumo	N/A

STEP-BY-STEP METHOD DETAILS

Feces and colonic tissue collection

Timing: 1.5 h

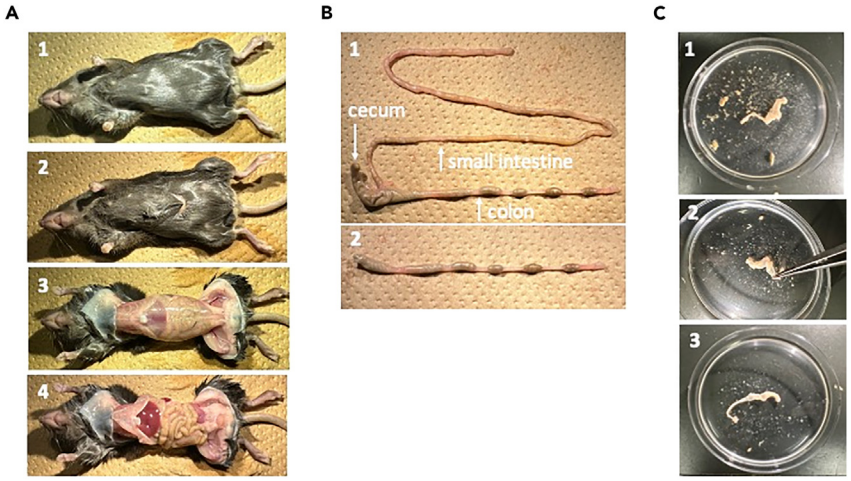

Figure 1. Dissecting a mouse and processing the tissues

These steps are to collect fresh feces and colonic tissues to be used for downstream analysis.

1. Collect fresh feces.
   a. Place mouse in a sterile container and await defecation.
   b. Collect using sterilized forceps. Those feces should be immediately preserved at –80°C, or frozen in dry ice temporarily before transporting.
2. Euthanize mice by means of euthanasia which are approved by the Institutional Animal Care and Use Committee.
3. Incise the abdomen and collect colonic tissue from the mice (Figure 1A).
   a. Spray 70% ethanol onto the abdomen and thorax entirely.
   b. Create a 5–10 mm of horizontal incision in the abdomen and peel back the skin to expose the peritoneum.
   c. Cut again. Hold each side of the incision and pull to open the abdomen.
4. Identify the cecum. Cut with scissors to pull out the intestinal tissue (Figure 1B).
5. Remove cecum and small intestine.
6. Cut and open the colonic tissue longitudinally by using a blunt ended scissor.
   a. Wash off fecal contents and mucus by immersing the colonic tissue in CMF PBS at room temperature.
   b. Transfer the colonic tissue to another CMF PBS to wash off again (Figure 1C).
7. Immediately move forward to the colonic tissue digestion step, or cool the tissue in ice-cold CMF HBSS/FBS before transporting (Figure 2A).

⚠ CRITICAL: To see the effect of NMP administration on microbiota, it is important to collect feces at pre-NMP treatment conditions, in addition to post-NMP treatment conditions, which will be stored at 80°C and subjected to 16s rRNA metagenomic analysis at the end. Colonic tissue can temporarily be transported in ice-cold CMF HBSS/FBS, but it should move to the following step as soon as possible.

Optional: In step 5, luminal contents can be collected for further microbiota analysis depending on the purpose. Keep in mind the microbiota composition of luminal contents can be different from that of feces.

Colonic tissue digestion

Timing: 2 h

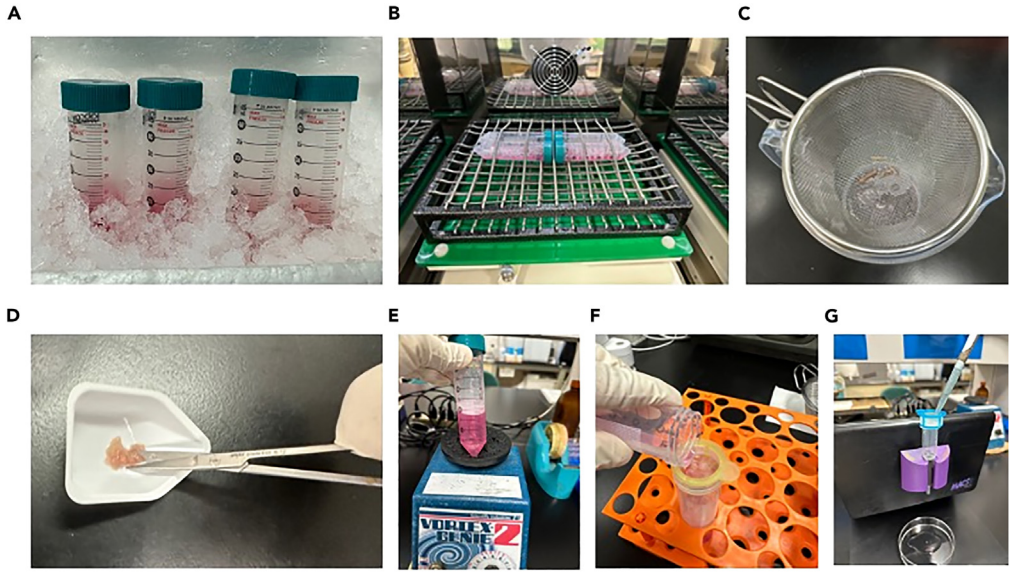

Figure 2. Digesting intestinal tissues and isolating immune cells

These steps to prepare the colonic tissue to be digested.

8. Prepare collagenase solution: 1.5 mg/mL Type IV Collagenase dissolved in pre-warmed CMF HBSS/FBS with 40 μg/mL of DNase at 37°C.
9. Cut the colonic tissue into approximately 1.5 cm pieces.
   a. Transfer it into separate 50 mL conical tubes with 30 mL of pre-warmed CMF HBSS/FBS and 2 mM EDTA.
   b. Use a single 50 mL conical tube for the pieces of colonic tissue per mouse.
10. Place each 50 mL conical tube horizontally in a pre-warmed orbital shaker (Figure 2B) and shake at 250 rpm for 20 min at 37°C.
11. Put a mesh wire strainer over a waste bucket and pour the contents of each 50 mL conical tube through to recover the 1.5 cm pieces of colonic tissues (Figure 2C).
12. Place the tissues in another 50 mL conical tube with 30 mL of pre-warmed CMF HBSS/FBS with 2 mM EDTA.
13. Repeat 3 and 4.
14. Pour the contents of each 50 mL conical tube through the wire strainer. Transfer 1.5 cm pieces of colonic tissues to a small plastic weigh boat.
15. Mince the 1.5 cm pieces of colonic tissues 10 times using scissors in the weigh boat (Figure 2D).
   a. Add minced tissues into 20 mL of collagenase solution.
   b. Place each 50 mL conical tube into an orbital shaker to digest at 200 rpm for 10–15 min at 37°C.
16. Vortex shortly to confirm sufficient dissociation of colonic tissues (Figure 2E). Filter through a 100 μm cell strainer into a 50 mL conical tube (Figure 2F).
17. Add 30 mL of ice-cold CMF HBSS/FBS and centrifuge at 440 G for 5 min at 4°C by using a floor-standing centrifuge.
18. Discard the supernatant to find a cell pellet at the bottom of the tube.
   a. Add 50 mL of ice-cold CMF HBSS/FBS.
   b. Repeat the centrifugation one more time.
19. Discard the supernatant. Use the cell pellet in the following steps.

Isolation of immune cells and RNA isolation

Timing: 2 h

These steps to isolate CD45+ colonic immune cells and purify RNA.

20. Prepare MACS buffer on ice by mixing MACS BSA stock solution and autoMACS rinsing solution (1:20).
21. Resuspend the cell pellet obtained from the previous step into the MACS buffer with CD45 MACS beads according to manufacturer's instructions.
22. Wash cells with MACS buffer and centrifuge at 440 G for 10 min at 4°C
23. Discard the supernatant and resuspend the cell pellet in 1 mL ice-cold MACS buffer and pass through a 100 μm cell strainer.
24. Enrich for magnetic bead-attached cells by positive selection using MACS LS magnetic column by passing through a 40 μm cell strainer (Figure 2G).
25. Wash cells with ice-cold CMF PBS and centrifuge at 440 G for 10 min at 4°C.
26. Repeat step 6 once more.
27. Discard the supernatant and resuspend the cell pellet with 350 μL of RLT buffer to lyse cells.
28. Purify RNA by using RNeasy Plus Micro kit according to the manufacturer's instructions.
29. Purified RNA was quantified by NanoDrop and preserved in a −80°C freezer until used for RNA-seq.

Optional: In step 8, TRIzol (Sigma-Aldrich) or other RNA-preserving reagents can be used instead of RLT buffer.

Pause point: After step 8, RLT buffer can be preserved at –80°C.

EXPECTED OUTCOMES

The estimated yield of isolated immune cell number will be more than 1.0 × 10^6 cells/colon with more than 90% cell viability. The RNA yield from them is about 0.2 μg.

LIMITATIONS

This protocol is for *in vivo* mouse model. Although this protocol aims to gain samples to evaluate the immune microenvironments by investigating the transcriptome of CD45+ immune cells, functional analysis for each immune cell population can be performed by flow cytometry analysis using those isolated cells (See also Geem D et al.[3] and Harusato A et al.[4]).

TROUBLESHOOTING

Problem 1

Over-digestion or under-digestion may lead to substantially lower cell yield and higher cell death rates.

Potential solution

Since the collagenase activity may vary in each lot number, collagenase should be tested when purchasing a new lot. In the preliminary experiments, the concentration of collagenase as well as the duration of tissue digestion need to be examined to obtain enough cell yield.

Problem 2

Insufficient removal of fatty tissue attaching to the intestine may lead to substantially lower cell yield.

Potential solution

Detailed observation of intestinal tissue by stereomicroscope to remove fatty tissue could be helpful.

Problem 3

Too many numbers of samples may affect the cell viability since it may take much more time to process all the samples.

Potential solution

After collagenase digestion, ice-cold CMF HBSS/FBS should be added to the samples immediately so that the activity of collagenase is decreased.

Problem 4

As for 16s rRNA metagenome analysis, the interpretation of the data would be difficult without having adequate negative controls.

Potential solution

It is important to have negative controls without NMP treatment, in addition to pre-NMP treatment conditions. In that way, the impact of NMPs on microbiota can rationally be accessed.

Problem 5

After colonic tissue digestion, immune cells can be clogged in the process of cell purification using magnetic beads.

Potential solution

The use of cell strainers is recommended when the cell suspension is applied to magnetic columns.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Akihito Harusato (harup@koto.kpu-m.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any datasets.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

A.H. wrote the paper. W.S. provided critical comments on RNA isolation. H.A., Y.N., H.N., and Y.I. participated in critical discussion. All the authors contributed to the manuscript by providing feedback.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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