

Bacterial Synchronized Transfer Assays in Bone Marrow Derived Macrophages

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[Abstract] Merocytophagy (“mero”, Greek for partial; “cytophagy” for cell eating) is a process by which cells acquire microbes and cytosolic material through phagocytosis of a small portion of neighboring cells upon cell-cell contact. Cell-cell contact dependent transfer events can be assessed through co-incubation of differently labeled cells. With these assays, it is difficult to analyze the recipient cells by microscopy or bacterial burden within only recipient cells. Therefore, we established a synchronized transfer assay that allows for recipient cells to be isolated from donor cells following transfer events at a high purity. Here, we present this assay in context of bacterial infections and cytosolic cellular staining. With this protocol, mechanisms of cell-cell contact dependent transfer events and the events following merocytophagy can easily be investigated.

Keywords: Cell transfer, Bacteria transfer, Bone Marrow Derived Macrophages, Transfer assay, Merocytophagy

[Background] Phagocytic cells can acquire infectious material when microbes are extracellular or directly from infected cells (Steele *et al.*, 2016). This host mediated process allows for initiation of an effective cellular response to clear infection (Ahmed *et al.*, 2008). However, this process can be exploited by bacterial pathogens to expand their replicative niche (Ireton, 2013; Steele *et al.*, 2016). We previously found that cells can acquire microbes and cytosolic material from neighboring cells through phagocytosis of a small portion of the infected cell which does not kill the donor cell (Steele *et al.*, 2016 and 2019). This process known as merocytophagy (“mero”, Greek for partial; “cytophagy” for cell eating) provides a mechanism for the intracellular bacteria to disseminate and sustain infection (Steele *et al.*, 2016 and 2019).

To understand the mechanism of merocytophagy, we established a synchronized transfer assay that can be used to analyze a purified population of recipient cells following transfer events. Previously described transfer assays allow for the quantification of transfer events, whereas this assay allows for the analysis of the engulfed material. Specifically, engulfed material following a transfer event can be analyzed using microscopy or microbial burdens. Herein, we describe a detailed protocol of the synchronized transfer assay to analyze transferred bacteria and cytosolic dye.

Materials and Reagents

1. Serological Pipette (e.g. Thermo Scientific, catalog number: 14-387-163)
2. 250 ml filter unit, 0.2 micron PES membrane filter, sterile (VWR, catalog number: 10040-464)

3. 10 ml syringes (Fisher Scientific, catalog number: 201635)
4. Fisherbrand™ Easy Reader™ Conical Polypropylene centrifuge tube 15 ml (Fisher Scientific, catalog number: 05-539-12)
5. Fisherbrand™ Easy Reader™ Conical Polypropylene centrifuge tube 50 ml (Fisher Scientific, catalog number: 05-539-13)
6. Fisherbrand™ Microscope Slides, Frosted (Fisher Scientific, catalog number: 12-552-3)
7. Fisherbrand™ Non-Tissue Culture treated Petri Dishes with Clear Lid (Fisher Scientific, catalog number: FB0875712)
8. Serological Pipette Tips (Various Sizes) (Fisher Scientific, catalog numbers: 07-200-571, 07-200-574, 07-200-573, 07-200-575)
9. Fisherbrand™ Cell Strainer (40 μ M) (Fisher Scientific, catalog number: 22363547)
10. Matsunami Micro Cover Glasses, Round (18 mm diameter) (VWR™, catalog number: 10026-138)
11. Micropipette Tips (Various Sizes) (VWR, catalog numbers: 10017-062, 10017-064, 10017-66, 1007-068, 1007-092)
12. Corning Costar Flat Bottom Cell Culture Plates (Fisher Scientific, catalog number: 3513)
13. 26G BD Precision Glide needles (Fisher Scientific, catalog number: 148266A)
14. C57BL/6J Mice (Jackson Laboratory, Stock No. 000664)
15. Fetal Bovine Serum (FBS) (Atlanta Biologicals, catalog number: S10350)
16. *Francisella tularensis* subsp. *holarctica* inducible T6SS mutant (Steele *et al.*, 2019)
17. *Francisella tularensis* subsp. *holarctica* live vaccine strain (LVS) (Center for Disease Control, CDC)
18. Recombinant murine M-CSF (rmM-CSF) (Peprotech, catalog number: 315-02)
19. Remel™ Hemoglobin (Fisher Scientific, catalog number: R451402)
20. J774A-1 Macrophages (ATCC® TIB-67)
21. L929 fibroblasts (ATCC® CLL-1)
22. Gibco™ TrypLE Express Enzyme (1x) (Fisher Scientific, catalog number: 12-605-028)
23. L-Aspartic Acid (Arcos Organics, catalog number: 56-84-8)
24. DL-Isoleucine (Fisher Scientific, catalog number: A17521)
25. L-Leucine (Arcos Organics, catalog number: 12512-100)
26. DL-Methionine (Arcos Organics, catalog number: 59-51-8)
27. DL-Serine (Fisher Scientific, catalog number: A15184)
28. L-Tyrosine (Fisher Scientific, catalog number: J63511)
29. L-Cysteine (Sigma-Aldrich, catalog number C7602-25G)
30. Thiamine HCl (Arcos Organics, catalog number: 67-03-8)
31. Spermine HCl (Fisher Scientific, catalog number: 5677-5GM)
32. L-Arginine (Arcos Organics, catalog number: 74-79-3)
33. L-Histidine (Arcos Organics, catalog number: 71-00-1)
34. L-Lysine (Fisher Scientific, catalog number: 657-27-2)

35. L-Proline (Fisher Scientific, catalog number: A10199)
36. DL-Threonine (Fisher Scientific, catalog number: A10606)
37. DL-Valine (Arcos Organics, catalog number: 516-06-3)
38. Ammonium Chloride (NH₄Cl) (ACROS Organics, catalog number: AC423285000)
39. 4% Paraformaldehyde (PFA) (Fisher Scientific, catalog number: AAJ19943K2)
40. Bacto Agar (Fisher Scientific, catalog number: DF1040010)
41. Brain Heart Infusions (BHI, Fisher Scientific, catalog number: DF0037178)
42. Cell Trace Far Red (CTR) Dye (ThermoFisher, catalog number: C34564)
43. Cell Trace CFSE (CFSE) (ThermoFisher, catalog number: C34553)
44. CorningTM RPMI 1640 Medium (Mod.) 1x with L-Glutamine (Fisher Scientific, catalog number: MT10-040-CV)
45. Dulbecco's Modification of Eagles Medium (DMEM) with 4.5 g/L glucose, L-glutamine, sodium pyruvate (Corning Life Sciences, catalog number: 10-013-CM)
46. DPBS (with magnesium (Mg) and calcium (Ca) (Corning Life Sciences, catalog number: 21-030-CM)
47. DPBS (without magnesium (Mg) and calcium (Ca) (Corning Life Sciences, catalog number: 21-031-CM)
48. EDTA disodium salt (ACROS Organics, catalog number: AC409975000)
49. GibcoTM GlutaMAXTM Supplement (Fisher Scientific, catalog number: 35050-061)
50. GibcoTM Sodium Bicarbonate 7.5% solution (Fisher Scientific, catalog number: 25080-094)
51. Gibco Penicillin-Streptomycin (10,000 U/ml) (Fisher Scientific, catalog number: 15140-148)
52. HyCloneTM MEM Non-essential Amino acids (Fisher Scientific, catalog number: SH30238.01)
53. HyCloneTM Sodium pyruvate (Fisher Scientific, catalog number: SH30239.01)
54. Potassium Bicarbonate (KHCO₃) (Fisher Scientific, catalog number: P184-500)
55. Sodium Hydroxide (Fisher Scientific, catalog number: SS255-1)
56. Trypan Blue (MP Biomedicals, catalog number: 195532)
57. Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, catalog number H-1200)
58. Calcium Pantothenate (Arcos Organics, catalog number: 137-08-6)
59. Magnesium Sulfate (MgSO₄·7H₂O) (Arcos Organics, catalog number: 10034-99-8)
60. Iron (II) Sulfate Heptahydrate (FeSO₄·7H₂O) (Fisher Scientific, catalog number: A15178)
61. Potassium Phosphate Monobasic (KH₂PO₄) (Fisher Scientific, catalog number: P285-500)
62. Potassium Phosphate Dibasic Anhydrous (K₂HPO₄) (Fisher Scientific, catalog number: P288-100)
63. D-Glucose (Fisher Scientific, catalog number: A16828)
64. HCl
65. NaCl
66. Isovitalex
67. 10% FBS DMEM (see Recipes)
68. ACK lysis buffer (see Recipes)

69. Chocolate agar plates (see Recipes)
70. Chamberlain's defined medium (CDM) (Chamberlain *et al.* [1965]) (see Recipes)
71. L cell media (see Recipes)
72. Bone marrow differentiation media (see Recipes)
73. 0.5 M EDTA (see Recipes)
74. 10 mM EDTA in 1x DPBS (without Mg and Ca) (see Recipes)
75. 10x Glucose Salts (see Recipes)

Equipment

1. Blunt forceps (Fisher Scientific, catalog number: 08-875-8B)
2. T75 flasks (Fisher Scientific, catalog number: 130190)
3. T175 flasks (Fisher Scientific, catalog number: 130191)
4. Surgical Scissors (Fisher Scientific, catalog number: 08-951-5)
5. Micropipettes (e.g., Rainin, catalog numbers: 17008655, 17008650, 17008652, 17008653)
6. Benchtop Swing Bucket Centrifuge (e.g., Eppendorf, catalog number: 022625004)
7. Hemocytometer (e.g., Fisher Scientific, catalog number 02-671-6)
8. Shaking Water bath (e.g., Fisher Scientific, catalog number: S37368)
9. Humidified 5% CO₂ incubators set to 37 °C (e.g., NuAire, NU-5700)
10. Leica DM4000 upright fluorescence microscope
11. OD spectrometer
12. Light Microscope
13. Biosafety cabinet

Software

1. Leica Application Suite X (LAS X) (Leica microsystems, www.leica-microsystems.com), for microscopy analysis

Procedure

A. L929 cell conditional media preparation

1. Grow L929 fibroblasts to confluence in two T75 flasks using L cell media (Recipe 9). Incubate at 37 °C 5% CO₂ for approximately 2 days.
2. Detach cells by removing existing media and adding 4 ml of TrypLE to flask.
3. Incubate at 37 °C 5% CO₂ for 5 mins or until cells are completely detached.
4. Collect detached cells by adding 10 ml of L cells media to flask and transfer to 50 ml conical tube.
5. Centrifuge at room temperature at 250-300 x g for 5 min to remove TrypLE from media. Re-

suspend cells in L cell media.

6. Count cells by adding 10 μ l of trypan blue to 10 μ l of cells. Add 10 μ l of mixture to hemocytometer and count under light microscope.
7. Plate cells at a density of 2.5×10^5 cells in 75 ml L cell media in T225 flasks.
8. Rock flasks back and forth to get equal coverage of the entire flask bottom.
9. Incubate at 37 °C 5% CO₂ for 10-12 days (watch for cobblestone appearance of cells).
10. Remove media from all of the flasks and place into 50 ml conical tubes.
11. Centrifuge at 300 $\times g$ for 5 min at 4 °C.
12. To ensure thorough mixing of all collected media, combine all of the supernatants into one 50 ml conical tube.
13. Vacuum filter media through a 250 ml filter unit.
14. Aliquot 45 ml into 50 ml conical tubes.
15. Transfer tubes to -20 °C for long-term storage. Thaw media at 37 °C when needed for bone marrow macrophage differentiation.

B. Bone marrow derived macrophages preparation

Note: Protocol can be performed using J774A.1 macrophage (ATCC® TIB-67), if bone marrow derived macrophages are unavailable. If performing EEA-1 staining, do not allow cells to fix for more than 7 min or antibody will not stain well.

1. Euthanize mouse by CO₂ and cervical dislocation in accordance with IACUC protocol.
2. Remove femurs from mouse in a biosafety cabinet.

Note: Keep bones intact and clean bones of any excess tissue.
3. For each femur, cut each end using sterile scissors. Cut approximately 1 mm off of each head of the bone or until you see an opening large enough for a 26G needle.
4. With forceps, hold the femur over a 50 ml conical tube.
5. Prepare a 10 ml syringe with needle. Fill syringe with 10 ml of DMEM.
6. Insert needle into the opening of the bone.
7. Flush femur with 5 ml of DMEM into the 50 ml conical tube.

Note: Be sure to stick the needle into each opening of the femur. If needle cannot enter the femur, cut more off of the end.
8. Repeat Steps B6 and B7 with second femur.
9. Filter the solution with a 40 μ m cell strainer into a fresh 50 ml conical tube to remove clumps of tissue.
10. Centrifuge cells at room temperature at 250-300 $\times g$ for 5 min.
11. Remove media and gently resuspend pellet in residual media (approximately 1 ml).
12. Add 1 ml of ACK lysis buffer (Recipe 2) and incubate for 60 s.
13. Add 9 ml of DMEM.
14. Centrifuge cells at room temperature at 250-300 $\times g$ for 5 min.
15. Remove media and resuspend cells in approximately 10 ml of L cell differentiation media

(Recipe 1 and Procedure A).

16. Seed approximately 5 ml in 20 ml final volume of media into non-tissue culture treated Petri dish.
17. Allow cells to differentiate in 37 °C 5% CO₂ incubator for 6 days.

Note: Ensure proper differentiation by checking morphology periodically. See Figure 1 for reference of proper differentiation (cells appear with elongated shape).

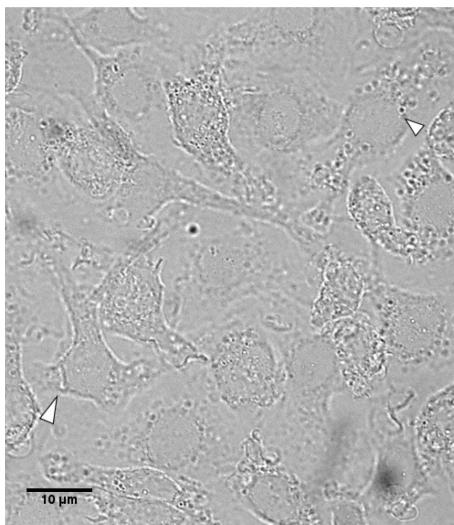


Figure 1. Morphology of Day 5 differentiated BMDMs. Appearance of elongated shape as indicated by arrowheads.

18. On Day 6, remove media from cells and wash cells by pipetting 10 ml of 1x DPBS (with Mg and Ca). Repeat wash 2 more times.
19. Following final wash, add 3-4 ml/plate of 10 mM EDTA in 1x DPBS (without Mg and Ca), pH 7.2-7.4.
20. Incubate plates at 37 °C for approximately 5 min or until cells begin to lift off.
21. Add 5-8 ml/plate of DMEM with 10% FBS DMEM (Recipe 5) and transfer to a 15 ml conical tube.
22. Centrifuge cells at room temperature at 250-300 x g for 5 min.
23. Remove media and resuspend cells in 10 ml of DMEM with 10% FBS.
24. Count cells using a hemocytometer.

Note: Expect a cell count of 6 to 8 million cells per plate.

25. Seed a 12-well tissue culture treated plate at 2×10^5 cells per well in a total volume of 1 ml per well.

Note: For each sample, seed 1 well with a coverslip (recipient sample) and 1 well without a coverslip (donor cells).

26. Incubate overnight at 37 °C 5% CO₂.

C. Transfer of cell permeable dye

Note: Wells should be set up with CFSE and CTR cells only (i.e. cells that do not have contact).

1. Stain donor cells with Cell Trace CFSE (CFSE) at a final concentration of 5 μ M in dPBS with Mg and Ca (500 μ l per well) in a 12-well plate.
2. Stain recipient cells with Cell Trace Far Red (CTR) at a final concentration of 1 μ M in dPBS with Mg and Ca (500 μ l per well) in a 12-well plate.
3. Incubate cells at 37 °C 5% CO₂ for 20 min.
4. Wash cells 3 times each with 1 ml of pre-warmed 10% FBS DMEM.
Note: 10% FBS DMEM should be pre-warmed in a 37 °C water bath for 20 min.
5. After the final wash, pick up the coverslip with recipient cells and invert it onto the well with donor cells so that the cells are in direct contact.
6. Incubate for 30 min at 37 °C 5% CO₂.
7. Remove the coverslip and return it into the original well in its original orientation.
8. At desired time point, add 4% PFA to coverslip (1 ml per well) and incubate at room temperature for 7 min (for EEA-1 staining) or 10 min for other antibodies.
9. Wash coverslips 3 times with 1 ml of 1x DPBS (with Mg and Ca).
10. Perform desired microscopy staining (e.g., LAMP-1 [Steele *et al.*, 2019]).
11. Wash coverslips 3 times with 1 ml of 1x DPBS.
12. Mount coverslips onto slides using mounting media by adding approximately 10 μ l of mounting media to slides and inverting coverslip onto slide.

D. Bacterial infection

Note: Various bacteria can be used for this assay with modifications needed for growth and infection conditions. Control wells should be set up with CFSE and CTR cells only.

1. Streak out *Francisella tularensis* freezer stock on chocolate agar.
2. Incubate plate at 37 °C for 3 days.
3. Pick 4-6 individual colonies from the plate and suspend colonies in 3 ml of CDM.
4. Incubate culture overnight at 37 °C in a shaking water bath.
5. Next day, spin down 2 ml of culture at room temperature at 20,000 \times g for 2 min.
6. Remove the supernatant and resuspend each pellet in 1 ml of PBS.
7. Blank OD spec with 1 ml of PBS.
8. Measure OD₆₀₀ of culture (desired OD should be 0.34-0.36, if not incubation should continue).
9. Infect donor cells at MOI 100 in 250 μ l total volume of 10% FBS.
10. Incubate for 2 h at 37 °C 5% CO₂.
11. Add 10 μ g/ml of gentamicin in a total volume of 500 μ l per well.
12. Twenty hours following inoculation of bacteria, replace media on all samples.
13. Invert coverslip of recipient cells onto donor cells to allow for direct contact.
14. Incubate for 30 min at 37 °C.
15. Remove the coverslip and put it into the original well in its original orientation.
16. At desired time point, add 4% PFA to coverslip (1 ml per well) and incubate at room temperature for 7 min (for EEA-1 staining) or 10 min for other antibodies.

17. Wash coverslips 3 times with 1 ml of 1x DPBS (with Mg and Ca).
18. Perform desired microscopy staining (e.g., LAMP-1 [Steele *et al.*, 2019])
19. Wash coverslips 3 times with 1 ml of 1x DPBS.
20. Mount coverslips onto slides using mounting media by adding approximately 10 μ l of mounting media to slides and inverting coverslip onto slide.

Data analysis

Microscopy results were collected using a Leica DM400 upright fluorescence microscope. For each sample, 50 images were taken at each desired time point per experiment. Images were analyzed using Leica LAS X software. The number of cells that acquired material from donor cells can be determined by counting the number of recipient cells that obtained either bacteria or CFSE (Figure 2 for reference). Transwells (or physical separation) can be used to determine the number of phagocytosis events of extracellular material (Steele *et al.*, 2016 and 2019).

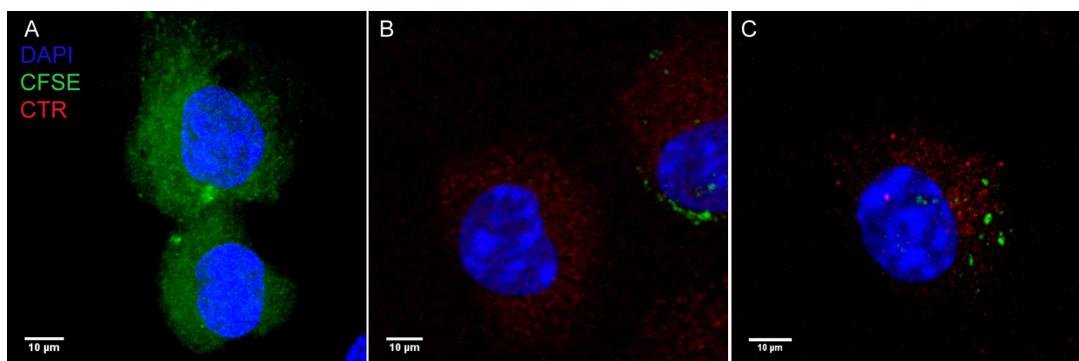


Figure 2. Representative microscopy of CFSE transfer events. A. CFSE stained donor BMDMs. B. CTR stained recipient BMDM. C. CFSE transfer to CTR stained recipient BMDM.

Recipes

1. Bone marrow differentiation media (150 ml for 1 mouse)
75 ml of DMEM
30 ml of heat inactivated FBS
45 ml L929 conditional media (can be substituted with M-CSF, Griffin *et al.*, 2013)
1.5 ml of 100 mM L-glutamine (or GlutaMax)
1.5 ml of 100 mM sodium pyruvate
1.5 ml of 7.5% sodium bicarbonate solution
Mix and filter sterilize
2. ACK lysis buffer
8.3 g of NH_4Cl
1.0 g of KHCO_3

200 μ l of 0.5 M EDTA

Add ddH₂O to 1 L, adjust to pH 7.2-7.4 and filter sterilize

3. 0.5 M EDTA

Dissolve 186.1 g EDTA disodium salt in 750 ml of ddH₂O

Add 15 g solid NaOH to solution to solubilize EDTA

Use 5 N NaOH to adjust pH to 7.5-8.0

4. 10 mM EDTA in 1x DPBS (without Mg and Ca)

Add 20 ml of 0.5 M EDTA to 1 L of 1x DPBS (without Mg and Ca)

5. 10% FBS DMEM

Add 100 ml of thawed heat inactivated FBS to 1 L of DMEM

6. Chamberlain's defined medium (CDM) (Chamberlain *et al.*, 1965)

Add components to a final concentration of 1x in a desired volume of H₂O:

L-Aspartic Acid (100x stock: 2 g into 50 ml HCl)

DL-Isoleucine (100x stock: 2 g into 50 ml HCl)

L-Leucine (100x stock: 2 g into 50 ml HCl)

DL-Methionine (100x stock: 2 g into 50 ml HCl)

DL-Serine (100x stock: 2 g into 50 ml HCl)

L-Tyrosine (100x stock: 2 g into 50 ml HCl)

L-Cysteine (100x stock: 1 g into 50 ml HCl)

Thiamine HCl (100x stock: 200 mg into 50 ml HCl)

Spermine HCl (100x stock: 200 mg into 50 ml HCl)

L-Arginine (100x stock: 2 g into 50 ml H₂O)

L-Histidine (100x stock: 1 g into 50 ml H₂O)

L-Lysine (100x stock: 2 g into 50 ml H₂O)

L-Proline (100x stock: 5 g into 50 ml H₂O)

DL-Threonine (100x stock: 2.5 g into 50 ml H₂O)

DL-Valine (100x stock: 2 g into 50 ml H₂O)

Calcium Pantothenate (100x stock: 100 mg into 50 ml H₂O)

10x Glucose Salts (see Recipe 7)

Once all components are added, pH solution to 6.2-6.4

7. 10x Glucose salts

25 g NaCl

338 mg MgSO₄·7H₂O

5 mg FeSO₄·7H₂O

2.5 g KH₂PO₄

2.5 g K₂HPO₄

10 g Glucose

250 ml H₂O

8. Chocolate agar plates

In a 2 L flask, add:

25 g of BHI

15 g of Bacto Agar

500 ml of H₂O

In a second 2 L flask add:

10 g of hemoglobin

500 ml of H₂O

Autoclave for 20 min on liquid cycle

Remove media from autoclave and place into a 50 °C water bath

Once cooled, add 10 ml of isovitalex

Combine hemoglobin to BHI and gently stir by hand

9. L cell media

500 ml of RPMI 1640

50 ml of heat inactivated FBS

2.5 ml of Pen/strep

5 ml of 100 mM sodium pyruvate

5 ml 100 mM non-essential amino acids

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Competing interests

No competing interests declared.

Ethics

All animals were handled according to approved institutional animal care and use committee (IACUC) protocol #4946 at Washington State University.

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