The RAW 264.7 cells used by the Alliance for Cellular Signaling (AfCS) were originally obtained from the American Type Culture Collection (ATCC; cat. no. TIB-71; lot no. 2263775). The vial of cells received from the ATCC was thawed using the protocol that follows. The thawed cells were expanded by maintenance in RAW 264.7 growth medium 1 (RAWGM1) and passage every two days until sufficient cells were obtained for preparation of frozen stocks. Numerous aliquots of frozen cells from similar passages provide a uniform source of RAW 264.7 cells for experimentation by AfCS laboratories. Frozen aliquots of cells from the AfCS contain approximately $5 \times 10^6$ cells in 1 ml of RAW 264.7 freezing medium (90% RAWGM1 and 10% dimethyl sulfoxide [DMSO], PS00000568). The thaw procedure described below should yield a starting culture of $5 \times 10^5$ RAW 264.7 cells/ml with a confluence of >50% in a 100-mm petri dish.

Murine macrophages and macrophage–like cell lines such as RAW264.7 adhere to tissue culture–grade plastic through cation-dependent integrin receptors and other cation-independent receptors, predominantly the murine scavenger receptors (MSRs) (Fraser, Hughes, and Gordon. [1993] Nature 364[6435]:343-346). Cells are maintained on tissue culture–treated petri dishes immediately after thawing for two passages to facilitate recovery. After two passages, cells are transferred to non–tissue culture–treated petri dishes (ultra-dish petri dishes) in order to reduce adhesion during routine culture. Adherence of macrophages to non–tissue culture–treated petri dishes is mediated by $\alpha_M\beta_2$ (CR3) integrins (Rosen and Gordon. [1987] J. Exp. Med. 166[6]:1685-1701) and is sufficiently weak that cells may be detached by the sheer force produced by media flowing over the cells. Use of cation chelators such as EDTA readily reverses these adherence properties and can be used to detach RAW 264.7 cells from either tissue culture–grade plastic or non–tissue culture–grade plastic. When the cells are first transferred to the non–tissue culture–treated petri dishes, they are also strongly adherent. This adherence on the petri dishes typically lasts for approximately three or four passages (~10 days). The thawed cells are released from both types of plates during this recovery period using versene solution (1X PBS/0.5 mM EDTA). Cells are cultured for at least 3 weeks prior to use for experimentation. During this 3-week recovery period, the doubling times will increase and plateau at $12.5 \pm 2$ hr (n=20). Concurrently, the level of adherence to the non–tissue culture–petri dishes will decrease. As adherence declines, passaging of cells can be done with medium alone.

Note: macrophages are extremely sensitive to lipopolysaccharide (LPS) endotoxin from Gram-negative bacteria. LPS has major effects on macrophage phenotype and function, including adhesion. All solutions, buffers, and media should be made with sterile, endotoxin-tested, distilled deionized water.
Thaw Procedure

1. Transfer RAW 264.7 growth medium 1 (RAWGM1) to a T75 flask (30 ml of RAWGM1 per flask).
2. Place flask in incubator at 37 °C with a 5% CO₂ atmosphere for at least 30 min to equilibrate medium. (Note: all manipulations and additions to cells are done with sterile tissue culture technique.)
3. Hold tube of frozen cells in a 37 °C water bath until fully thawed. Do not leave tube in water bath unattended.
4. Pipette cell suspension immediately into a 15-ml conical tube containing 9 ml of equilibrated RAWGM1.
5. Centrifuge tube of cells at 400 x g for 5 min at room temperature.
6. Remove all but approximately 5 µl of the supernatant.
7. Flick tube with an index finger to gently resuspend cell pellet.
8. Add 10 ml of equilibrated RAWGM1.
9. Pipette up and down gently to resuspend cells in the 10 ml of medium.
10. Transfer the suspension of cells to a 100-mm tissue culture petri dish.
11. Place petri dish containing cells in an incubator set to 37 °C and an atmosphere with 5% CO₂.
12. Enter barcode from the frozen stock vial into the cell line GUI.
13. After 48 hr, equilibrate 60 ml of RAWGM1 for at least 30 min.
14. Warm versene solution in a 37 °C water bath.
15. Tip petri dish containing cells at a 20 to 30 degree angle and aspirate the pooled medium from the lowest side of the dish.
16. Add 5 ml of warm versene solution gently to lowest side of dish, then place dish flat, ensuring liquid covers cells to wash cells and remove any residual medium.
17. Immediately tip plate at a 20 to 30 degree angle and aspirate the pooled solution from lowest side of dish.
18. Add 5 ml of fresh, warm versene solution, place dish flat in tissue culture hood (ensure that the liquid covers the cells), and incubate in hood at room temperature for 2 min.
19. Tip petri dish at a 20 to 30 degree angle and gently stream versene solution over the adherent cells using a 10-ml pipette. Rotate the plate 90 degrees. Repeat this process 3 or 4 times until all sections of dish are clear of cells. Rinsing will dislodge cells and create a single cell suspension. Avoid creating bubbles by not taking up and expelling air with the versene solution in the pipettes.
20. Transfer solution containing dislodged cells to a 15-ml conical tube.
21. Centrifuge at 400 x g for 5 min at room temperature.
22. Aspirate versene solution away from cell pellet.
23. Add 10 ml of equilibrated RAWGM1.
24. Gently pipette up and down to suspend the cells in the pellet.
25. Remove a volume of single cell suspension and dilute with trypan blue and versene solution for counting. (For example remove 10 µl of single cell suspension and transfer to a microfuge tube containing 90 µl of versene.
solution and 100 µl of trypan blue. Gently mix the 1 to 20 dilution of cells by pipetting).

26. Count the dilution of white and blue cells using a hemacytometer.
27. Enter counts into the cell line GUI that is used to calculate the number of cells and the volume needed to seed new vessels.
28. Add volume of equilibrated RAWGM1, calculated by the cell line GUI, to a new tissue culture–treated vessel (determined to give 10 ml/100-mm dish and 30 ml/150-mm dish).
29. Pipette cell suspension up and down, without introducing bubbles, to resuspend settled cells.
30. Transfer volume of RAW 264.7 cells, calculated by the cell line GUI (to yield $1 \times 10^6$ total cells/100-mm dish and $3 \times 10^6$ total cells/150-mm dish) to dish with fresh media.
31. Pipette up and down gently 2 to 4 times to mix and evenly distribute cells in dish.
32. Place dish carefully in a humidified incubator set at 37 °C with an atmosphere containing 5% CO₂.
33. Steps 28 through 32 can be repeated to seed multiple dishes.
34. Aspirate any residual suspended cells for disposal.

**Reagents and Materials**

RAW 264.7 growth medium 1 (RAWGM1): AfCS Solution Protocol ID PS00000510

Ultra-dish petri dish (standard), 100 mm x 15 mm: Midwest Scientific; catalog no. 900

Ultra-dish petri dish (standard), 150 mm x 15 mm: Midwest Scientific; catalog no. 902

Versene solution: Invitrogen; catalog no. 15040066

Trypan blue solution: Sigma-Aldrich; catalog no. T8154

Hemacytometer: Fisher Scientific; catalog no. 02-671-5

Falcon tissue culture dish, 100 mm x 20 mm: BD Biosciences; catalog no. 353003

Corning cell culture dish, 150 mm x 25 mm: Corning Inc.; catalog no. 430599

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