# REAL-TIME SPECTROFLUOROMETRIC PHAGOLYSOSOMAL FUSION ASSAY

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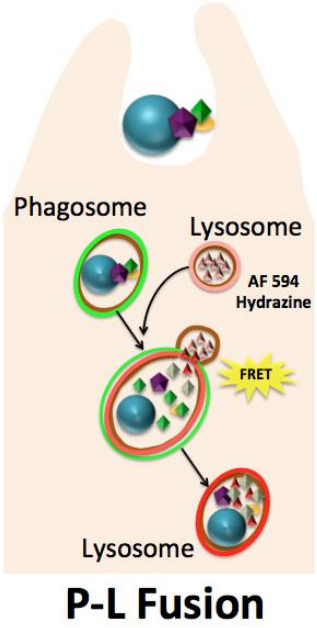
## BACKGROUND

The phagosome gains bactericidal properties by dynamically interacting with the endo/lysosomal compartments of the cells. The motor complex mediates phagolysosomal fusion by transporting the phagosome along the microtubule network towards the lysosomal compartment. This is not a single fusion event between a phagosome and a single lysosome. The phagosome progressively obtains the membranous and luminal components of a phagolysosome through multiple transient interactions with late endosomal and lysosomal compartments. Herein, we outline a real-time population-based spectrofluorometric assay that allows one to capture and quantify the kinetics of the accumulation of lysosomal constituents within the phagosome.




## EQUIPMENT

- Accutase [Sigma-Aldrich #A6964]
- Cell lifter
- Trypan blue and hemocytometer
- Falcon™ 96-Well Flat-Bottom Tissue Culture Treated Microplate (Black with Clear Bottom) [VWR #353219]

- Alexa Fluor™ 594 Hydrazide [Thermo Fisher #A10438]
- 1 M HEPES
- Dextrose
- FBS
- PBS
- Spectrometer
- RPMI
- Concanamycin A [Sigma-Aldrich #80890-47-7]

# PROTOCOL

**Day 0: Preparing for the Assay**

Preparation of particle-restricted donor Fluor:
***Refer to "MANNOSYLATED INTRAPHAGOSOMAL ASSAY PARTICLE PREPARATION" Protocol**

Loading macrophages with hydrazine:

1. For a 150 mm plate containing MØ, add 10 mL of accutase solution for 10 minutes at 37˚C and 5% $CO_2$.
2. Using a cell lifter, gently lift residual cells adhered to the plate.
3. Place the cell suspension into a 50-mL falcon tube containing 20 mL of warm DMEM – 10 media.
4. Centrifuge at 500g for 5 minutes at 4˚C.
5. Resuspend the pellet in R10 media + LCM and determine the concentration of cells using trypan blue and a hemocytometer
6. Aliquot 200 000 macrophages into each well of a 96-Well Flat-Bottom TC Microplate. (Black with Clear Bottom)
    a. This concentration of cells/well have been optimized for signal strength and monolayer density **(See Supplementary figure 1)**
7. Allow cells to adhere to the plate (2 – 3 hours)

8. Prepare AF594 hydrazine 50 µg/mL in RPMI and filter the mixture using a 0.22 µm filter and a syringe **(Minimize exposure to light by wrapping tubes in tin foil)**
    a. *Preparation of stock:* Dissolve the AF594 hydrazine powder in 1mL RPMI for a stock concentration of 1 mg/mL. Perform a 1/20 dilution to obtain the working concentration of 50 µg/mL.
9. Remove the R10 + LCM media from the wells
10. Add 200 uL of hydrazine to each well and wrap the 96 well plate in tin foil. ("Pulsing" the cells)
    a. Ensure there is a control well containing macrophages not loaded with hydrazine
11. Place the plate in the incubator for 18 hours.

**Day 1: Conducting the assay**

12. Remove the hydrazine from each well and aliquot into a 50 mL falcon tube covered in tin foil.
    a. *Hydrazine may be re-used for future experiments. You must filter (0.22 µm) the used hydrazine to remove any cellular particulates that may interfere with the experiment. However, keep in mind that the fluorescence is reduced with every use. Avoid using the same hydrazine more than twice.*
13. Add 200 uL of R10 + LCM media into each well and place the plate into the incubator for 4 hours. ("Chasing" the cells)
14. Make the assay binding buffer by adding 1 mL 1 M HEPES, 0.09 g Dextrose, and 5mL FBS to 100 mL of PBS. This should be made fresh the day of the experiment.
15. Count the stock of particle restricted donor fluora
    a. Dilute the stock 1:100 in PBS and count using the hemocytometer. (Account for the dilution factor when calculating the concentration of beads)
16. Prepare $1x10^{7}$beads/mL in assay binding buffer

17. Remove the R10 + LCM media from each well and gently wash each well with 200 uL PBS
18. Add 85 uL of 1x10 $^{7}$beads/mL to three empty wells for future background reads
19. Prepare 100 nM concanamycin A. [Inhibitor of v-ATPase, prevents phagolysosomal fusion]
    a. Dissolve the concanamycin A powder in DMSO to a stock concentration of 100 uM and make 10 uL aliquots. Store at – 20˚C.
    b. Add 10 uL of 100 uM to 9990 uL of PBS (1:1000 dilution) to prepare 100 nM concanamycin A. Vortex well. **(Supplementary figure 2)**
20. Set the spectrometer to 37 ˚C.
21. Remove the PBS from the experimental wells.
22. Add 200 uL of concanamycin A to the negative control wells containing macrophages loaded with hydrazine. Incubate at 37 ˚C for 10 minutes and protect from light.
    a. This incubation can be done in the spectrometer in order to read the background during this period.
23. Add 200 uL of assay binding buffer to the other experimental wells of the plate (excluding the negative control and the bead-only control wells)
24. During the 10 minute incubation period, three background fluorescent measurements are taken:
    a. **Excitation 594 nm| Emission 620 nm** – This measures the acceptor fluor emission reading in order to check for equal loading of AF 494 hydrazine. A large discrepancy in signal between wells may indicate inequities in monolayer density or unequal loading of hydrazine. In order to minimize variability, avoid exposure of the plate to light and mix the cell mixture well prior to aliquoting them to the wells.
    b. **Excitation 488 nm| Emission 520 nm** – This is to determine the background values of the donor fluora in the monolayer and in the bead control wells.
    c. **Excitation 488 nm| Emission 620 nm** – This is to determine the background values of FRET in the monolayer and in the bead only control wells.

i. This represent the proportion of the "FRET" signal that is due to signal bleed-though contributed by the beads themselves. This value is utilized in future calculations.

25. Remove the concanamycin A and assay binding buffer from the cells
26. Add 85 uL of beads to the experimental and negative control wells. Incubate at 37 ˚C for 10 minutes.
27. Remove the suspension containing unbound beads and add 100 uL assay binding buffer to every well.
28. Read the donor (Excitation 488 nm| Emission 520 nm) and FRET (Excitation 488 nm| Emission 620 nm) emission at 37 ˚C every minute for 3 hours. This allows for equilibrium of lysosomal constituent accumulation within the phagosomes.

## ANALYSIS

1. Calculate the Relative FRET Unit (RFU): $F_S/D_S - F_B/D_B$ for each well.
   a. $F_S$=FRET emission (Excitation 488 nm| Emission 620 nm) of the experimental sample
   $D_S$=donor emission (Excitation 488 nm| Emission 520 nm) of the experimental sample
   $F_B$="FRET" signal contribution of the beads only control well
   $D_B$=donor emission of the beads only control well[1]
2. Plot the RFU against time to generate the graph.

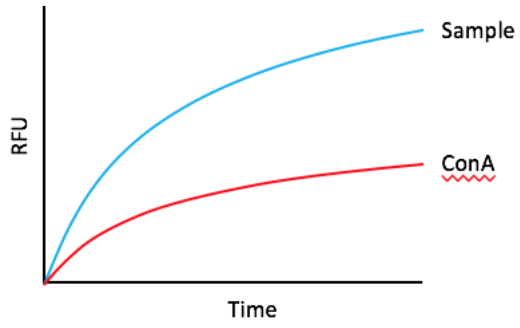


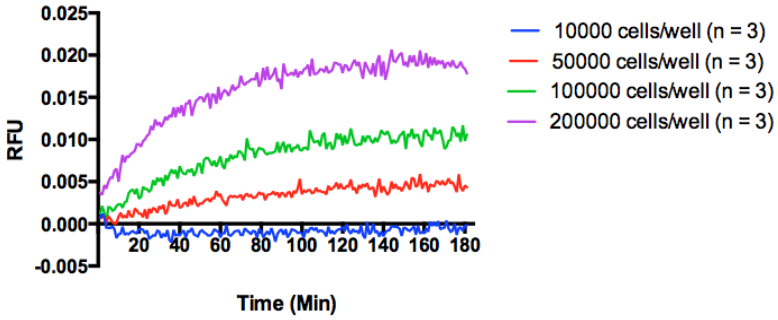

*Illustration of the expected graph*

3. Calculating the rate of Phagolysosomal fusion:
    a. Calculate the average RFU from 0 – 4 minutes and 28 – 32 minutes.
    b. Subtract the former from the latter.

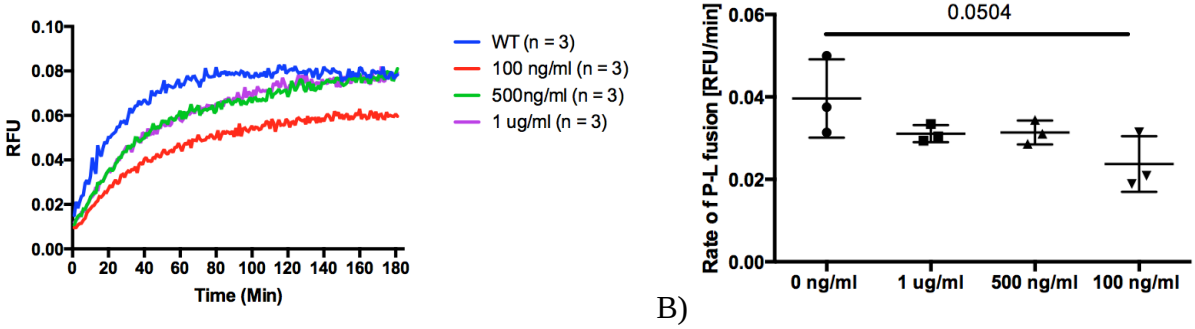
## LINKS AND REFERENCES


1. Yates RM, Hermetter A, Russell DG. The kinetics of phagosome maturation as a function of phagosome/lysosome fusion and acquisition of hydrolytic activity. *Traffic*. 2005;6(5):413-420. doi:10.1111/j.1600-0854.2005.00284.x.


## SUPPLEMENTAL FIGURES




**Supplemental Figure 1** *Optimization of the number of cells per well utilized in the real-time spectrofluorometric intraphagosomal assay detecting P-L fusion (FRET).* A) Average reading of RFU over 3 hours. The formation of a monolayer was confirmed visually in wells containing 10,000, 50,000, 100,000, 200,000 cells.




A) B)

**Supplemental Figure 2** *Optimization of the concentration of ConA in the inhibition of the rate of P-L fusion.* A) Average reading of RFU over 3 hours B) The rate of P-L fusion from 0 – 30 minutes. Treatment of cells with 100 ng/mL ConA resulted in the greatest impairment of P-L fusion.