Sweet Surprises: Unraveling the Sugary Side of Notch and How Overexpressed Fringe Enzymes Shape Osteoclastogenesis

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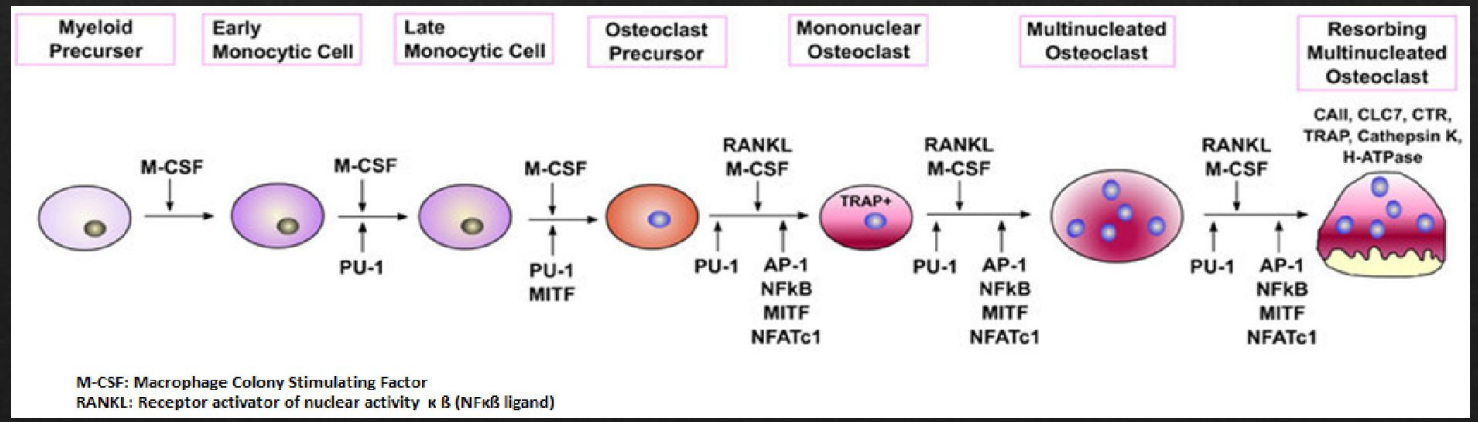
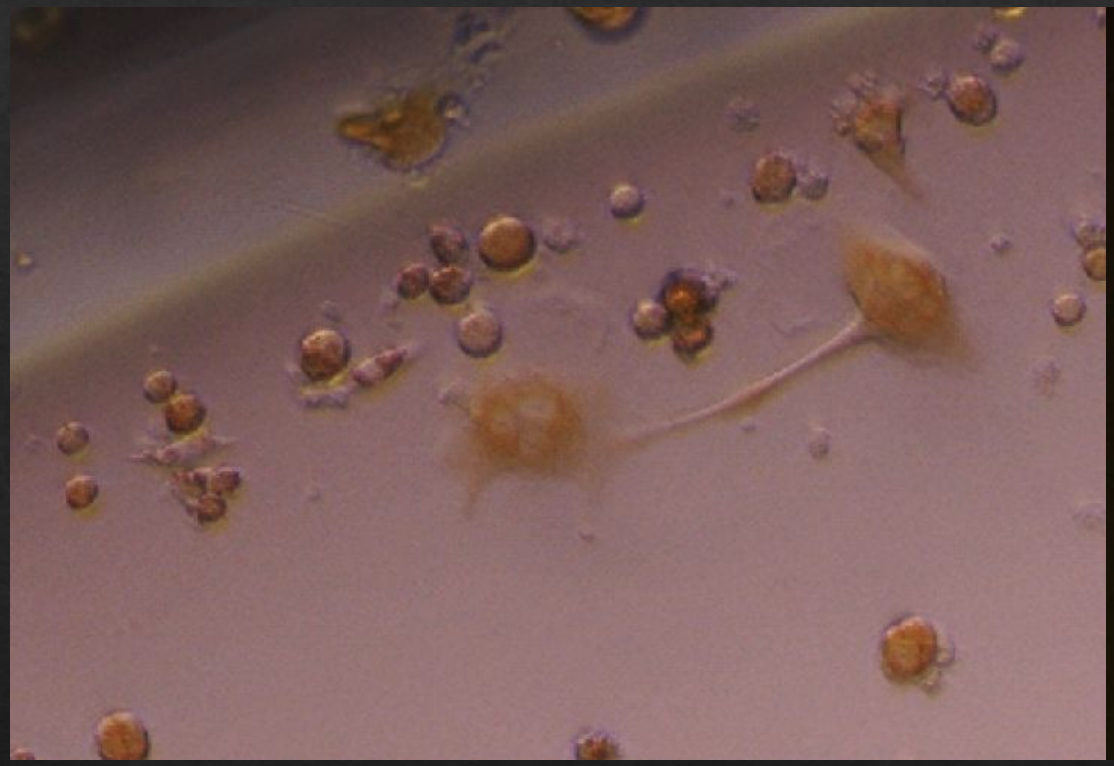
Background Information

- **Osteoclast** cells are large multinucleated phagocytic cells that play a crucial role in the resorption or breakdown of bone tissue in addition to their role in bone remodeling, growth, and repair.
- **Osteoclastogenesis** is dependent on RANK/RANKL pathway signaling while other pathways, such as Notch signaling, influence other factors of the cell life such as size, activity, and lifespan.
- Fringe enzymes are a specific *N*-acetylglucosaminyltransferase that extends *O*- fucose monosaccharides which modulates Notch pathway activity. Within mammals there are 3 homologs: Lunatic, Manic and Radical Fringes.

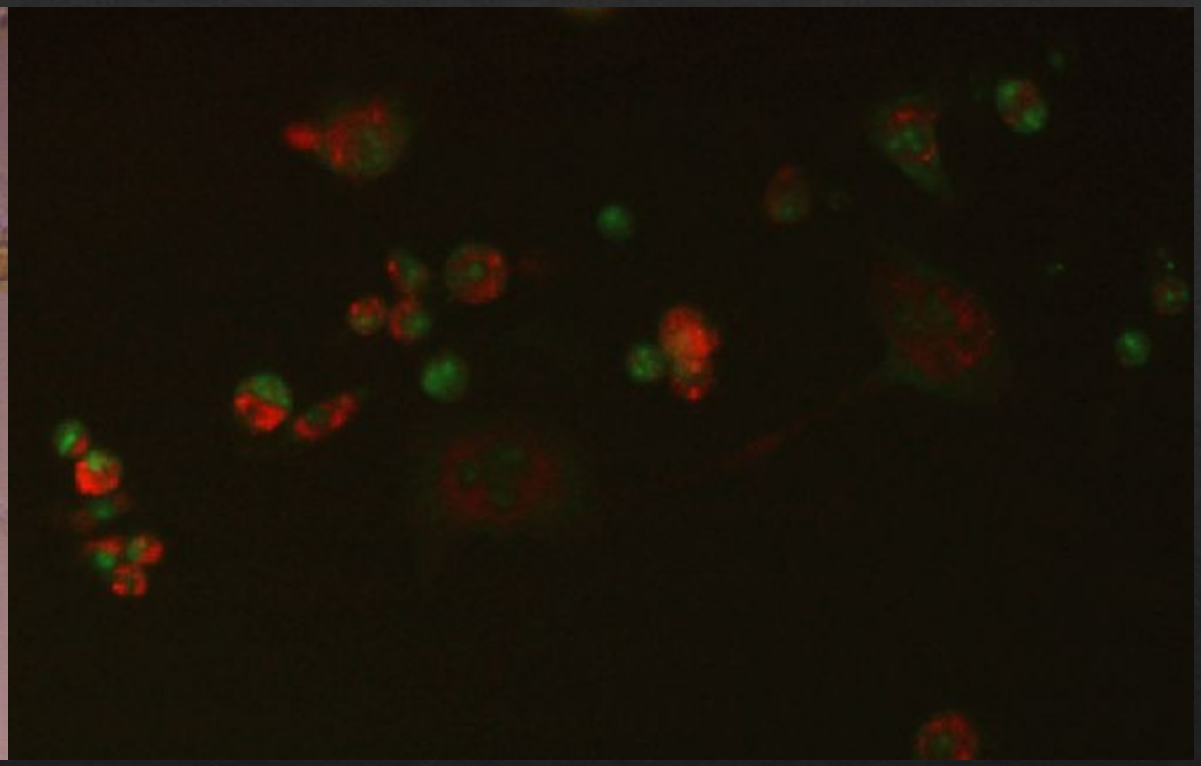


Stages of osteoclast differentiation from lineage cells [Soltanoff, et al. 2009]

Osteoclast Are Multinucleated Cells



Brightfield
Staining



Acridine Orange

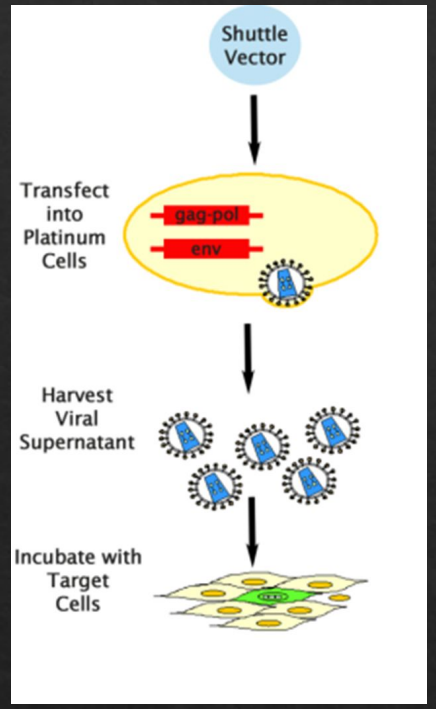
Experiment Overview

- Experiment 1
  - Impact of time on osteoclast formation within RAW264.7 cell lines [cultures fixed at 3 or 4 days after exposure to RANKL]
  - Control: EFGP fluorescing plasmids or M-Cherry fluorescing plasmids
- Experiment 2
  - Overexpression of Fringe glycosyltransferase within mouse BMM differentiation
  - Plating methods: Direct seeding into 96 well plates or transferring from 60mm petri dishes to 96 wells
    - Direct seeding is less stressful to the delicate precursor cells but does not allow for accurate cell numbers
    - 60mm transferred methodology tested by exposing virus for 24hrs or 6 hrs.

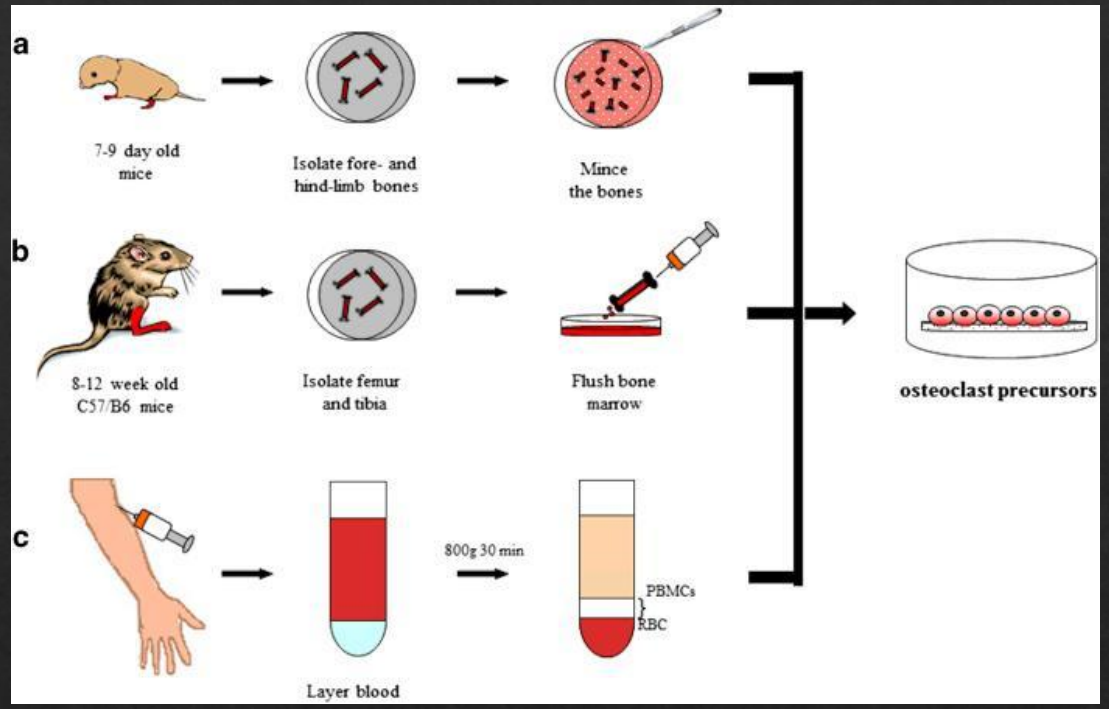
Methodology Overview

- Creating Overexpression:
  - Specific plasmids when introduced to a macrophage can overexpress (OE) Fringe enzymes. Viral plasmids are created with PLAT-E packaging cells infected with cDNA and membrane transgenes. After 48hrs the media supernatant is collected and stored for later infection of macrophages.
- Cell Lines:
  - **RAW264.7** are immortal mouse cells established from a male mouse tumor. **cultured** the cell lines for several passages, **infected** with plasmids, **selected** with puromycin antibiotic, **seeded** into 96 well plates, then **differentiated** with RANKL. After cells are **stained** with Acridine Orange, **fixed** and then **stained** for TRAP (Tartrate Resistant Acid Phosphate) activity.
  - **Primary bone marrow macrophages** are mouse cells **collected** from male mice femurs and tibias, **isolated** through culturing techniques, **seeded** in either 96 well plates (50,000 cells/well) or 60 mm petri dishes with M-CSF, **transfected** with viral plasmids, 60mm cultures **seeded** into 96 well plates with (10,000 cells/well), **selected** with puromycin antibiotic, and **differentiated** with RANKL.

Methodology Overview



Plasmids within the viral supernatant are crystallized within calcium phosphate microscopic crystals to shuttle plasmids within the macrophages to select for OE transgenes.
[Cell Biolabs, 2008]



Osteoclast precursor collection methods vary across species analyzed. In animal studies the precursors are collected from the bone marrow directly. [Marino, et al. 2014]

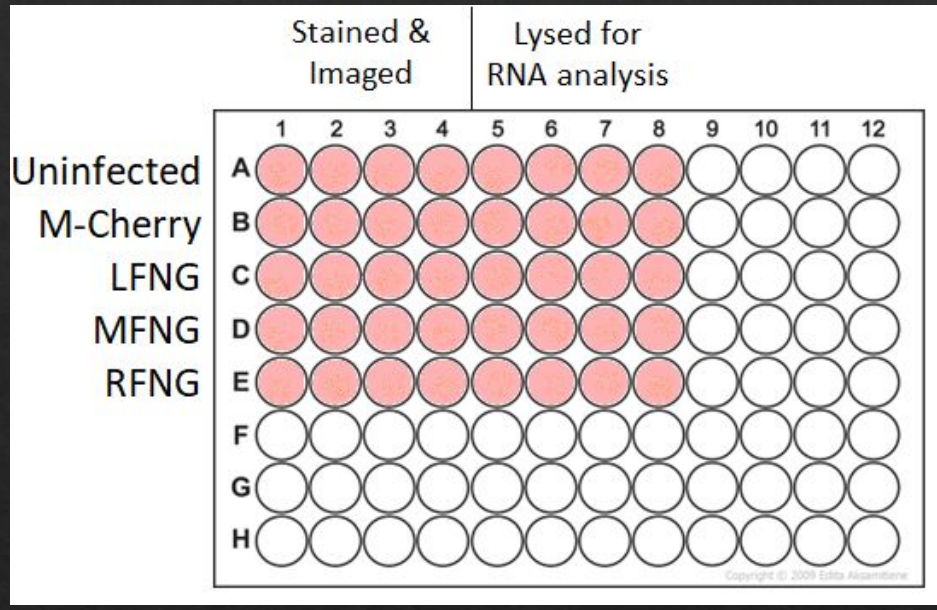


Diagram of 96-well plate used to analyze effectiveness of osteoclastogenesis within primary BMM

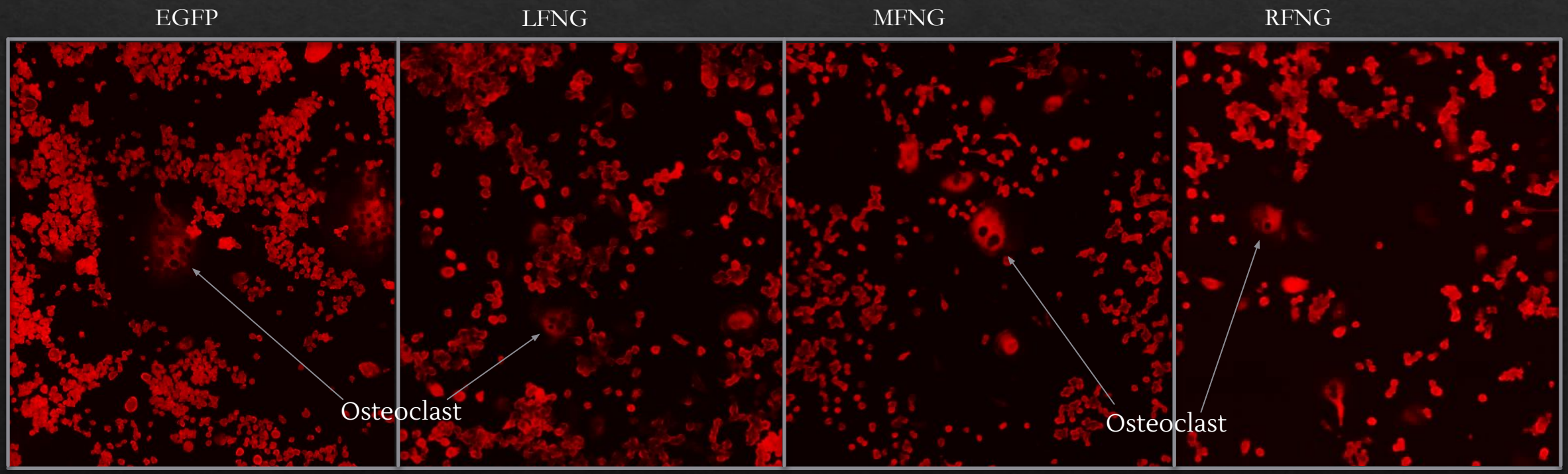
Results of Experiment 1

3 Days

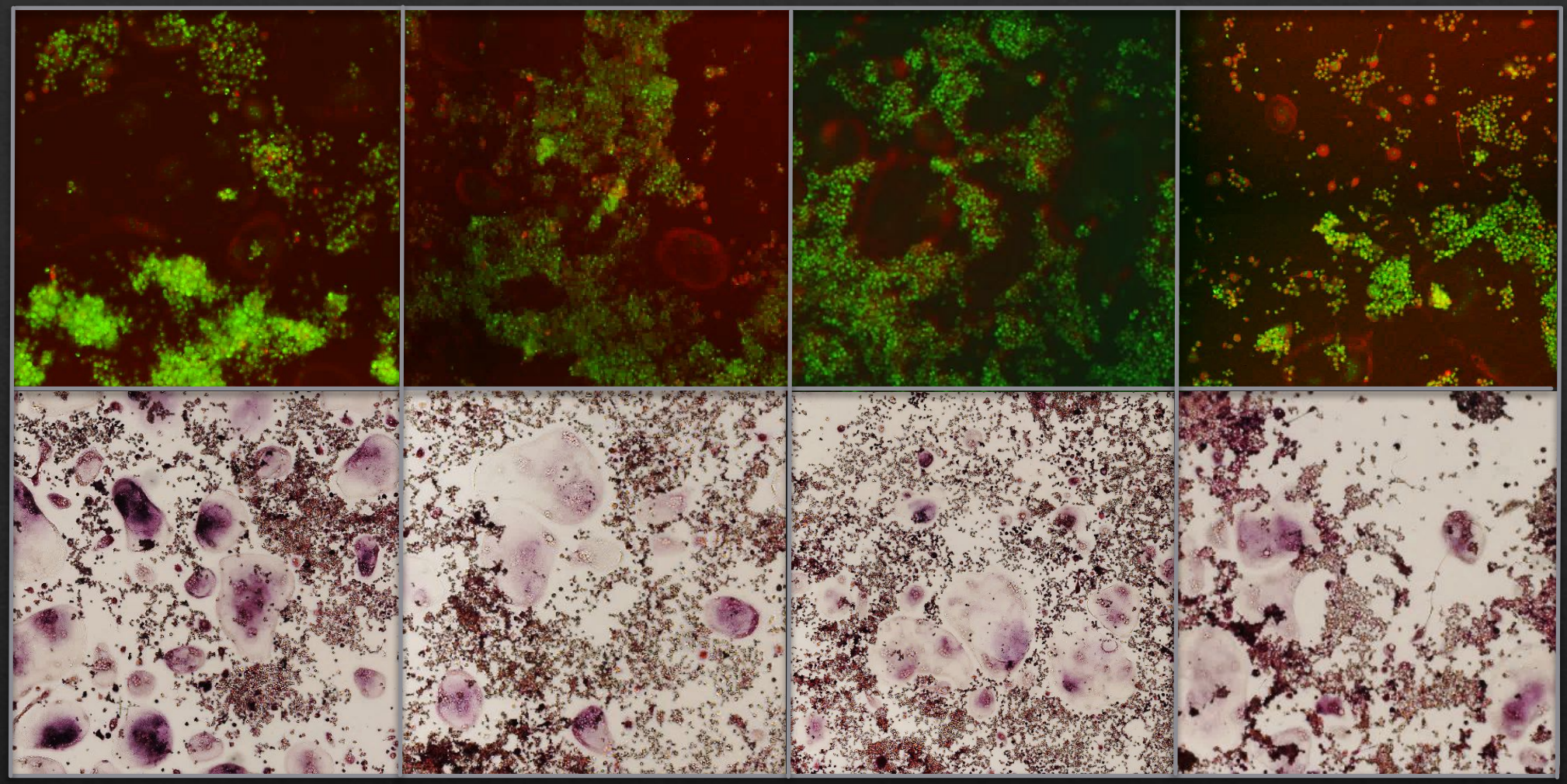
- Osteoclastogenesis is reduced in all cultures
- Osteoclasts that did form are smaller with less nuclei

4 Days

- Osteoclasts increase in number within culture
- Larger osteoclasts with more nuclei, morphological differences within Fringe lines



TRAP under Fluorescence (Texas Red) at 3 Days after RANKL exposure to induce differentiation



Control (EGFP)

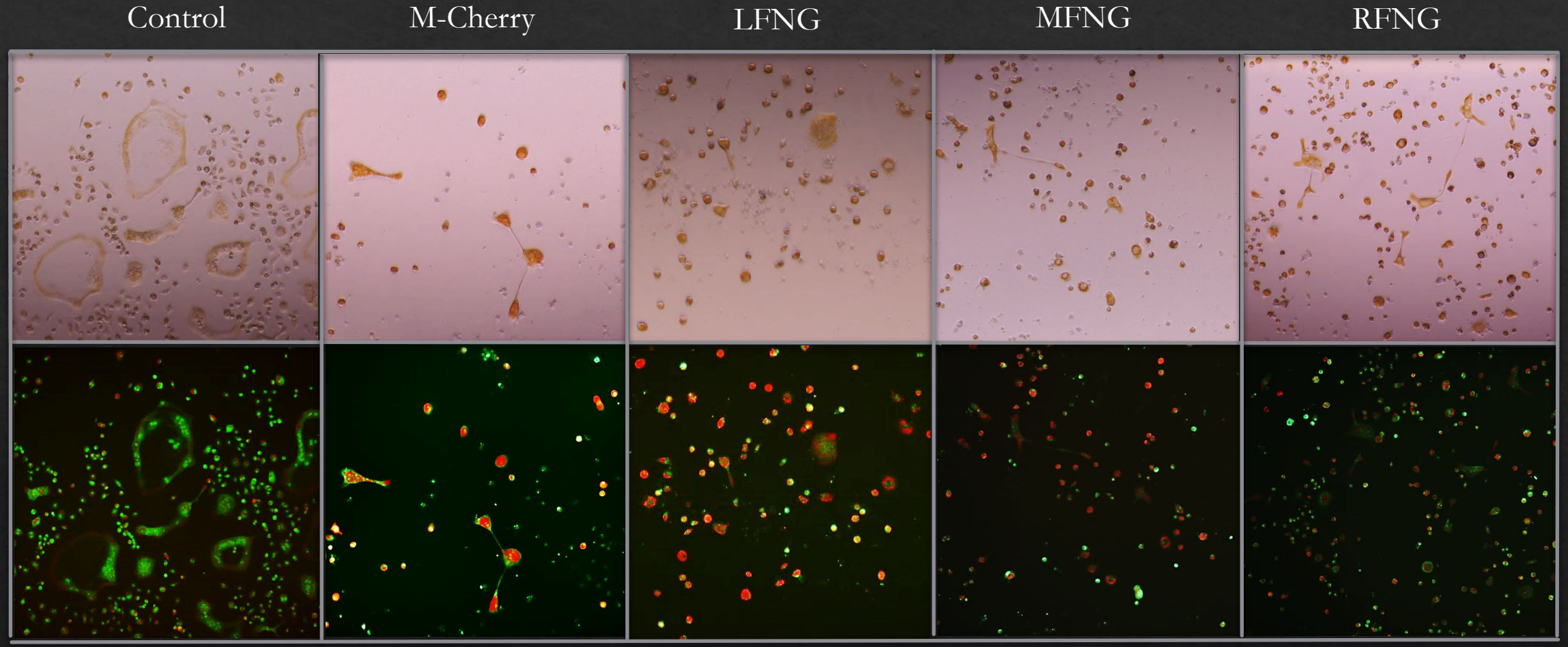
LFNG: large active osteoclasts at time of fixing

MFNG: larger osteoclasts with reduced function at time of staining indicating early cellular death

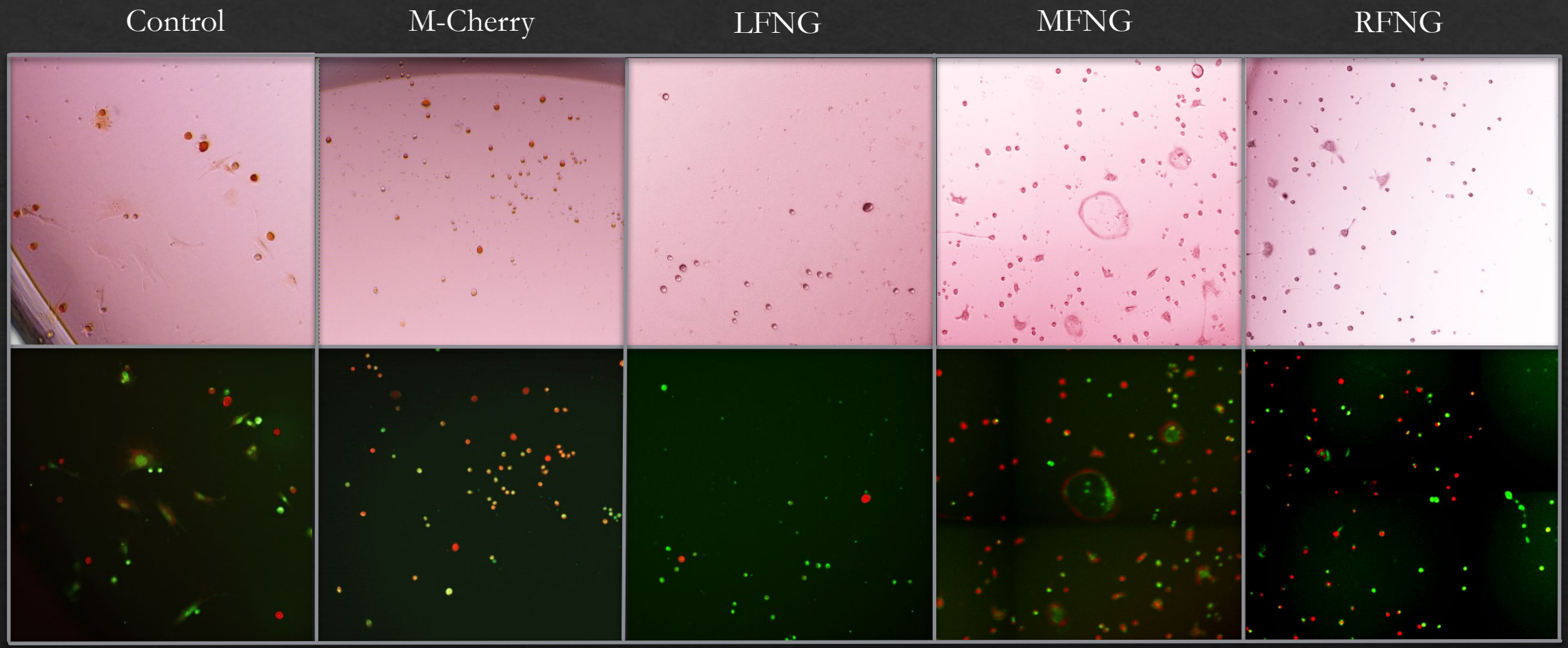
RFNG: fewer osteoclasts that presented smaller

Results of Experiment 2

96 well direct seeding



60mm Transfer to 96-Well



Discussion

- Experiment 1
  - Fringe homologs impact cell lifespan and morphology
  - Fluorescent plasmids as control interfere with staining.
- Experiment 2
  - Directly seeding BMM cells into 96 well plates and then transfecting showed the most notable impact to survival of transfection process with the densities tested
  - Exposing virus for 6 hours preserved the naïve state of the precursor macrophages, but alternative methods must be explored for higher survival rates during the transfer to 96 well plates
- What's Next?
  - Analysis of collected RNA from primary BMM to quantify Fringe OE
  - Test varying densities of direct seeding to 96 well plates
  - Knockout of Fringe homologs within RAW264.7 and primary BMM cell lines

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Selected References

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