SAND2022-14751C

Sandia National Laboratories

# Genetic Engineering for Bio-inorganic Materials

Kimberly Butler

The Rio Grande Symposium on Advanced Materials

October 24, 2022

U.S. DEPARTMENT OF ENERGY | NNSA

Sandia National Laboratories is a multimission laboratory managed and operated by National Technology & Engineering Solutions of Sandia, LLC, a wholly owned subsidiary of Honeywell International Inc., for the U.S. Department of Energy's National Nuclear Security Administration under contract DE-NA0003525.

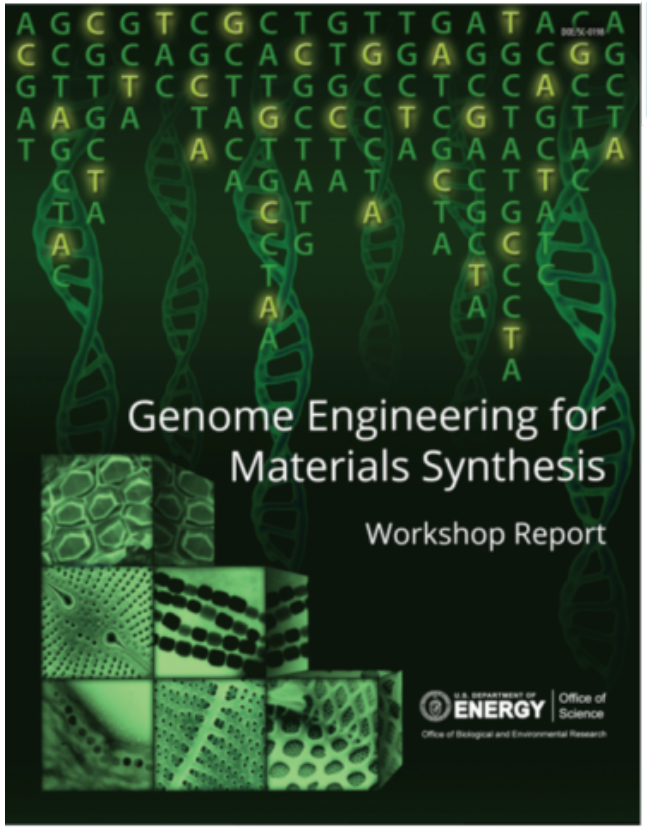
Sandia National Laboratories is a multimission laboratory managed and operated by National Technology & Engineering Solutions of Sandia, LLC, a wholly owned subsidiary of Honeywell International Inc., for the U.S. Department of Energy's National Nuclear Security Administration under contract DE-NA0003525.

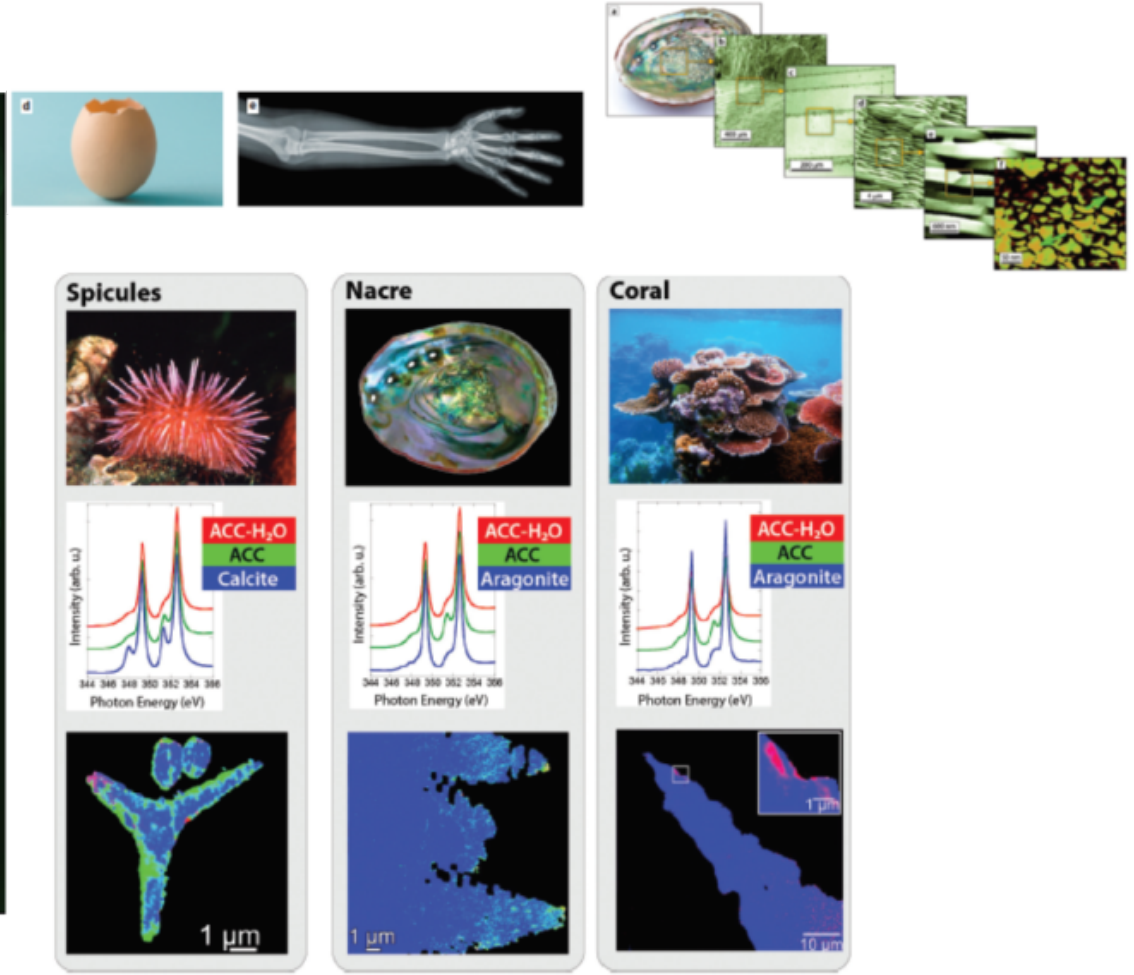
# Why engineer bio-inorganic synthesis?

Biological systems can synthesize complex materials with desired properties

- Fracture resistant
- Light weight
- Inherent sense and repair

Without toxic chemicals or high energy cost

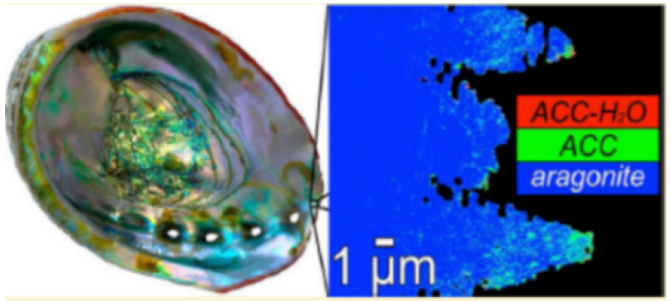


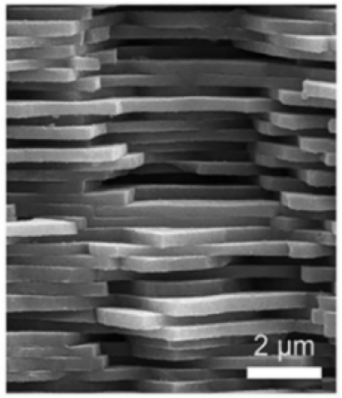

U.S. DOE. 2019. *Genome Engineering for Materials Synthesis Workshop Report*, DOE/SC-0198, U.S. Department of Energy Office of Science.

There is currently only one genetically tractable system, mammalian bone, for engineering macroscale bio-inorganic structures.

# Focus on nacre




DeVol et. al. *JACS*. 2015. 137:13325-33.
Peng et. al. *Matter.* 2020. 2:220-32.




Nacre: tough, exceptional fracture resistance

## Mollusk nacre organization

| Prismatic | Laminar | Crossed | Crossed-Lamellar | Isolated | Homogenous |
|---|---|---|---|---|---|

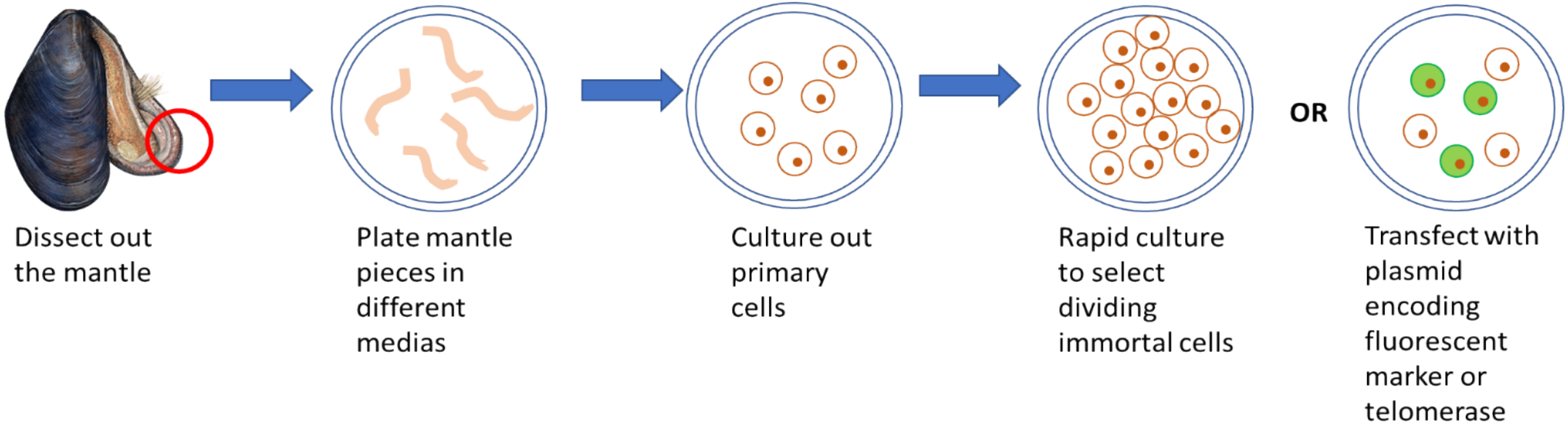
Identified 4 species of farmed mollusk with laminar nacre
Red Abalone
Ezo Abalone
Eastern Oyster
Blue mussel (aka PEI mussel)

# There are currently no cell lines that make nacre

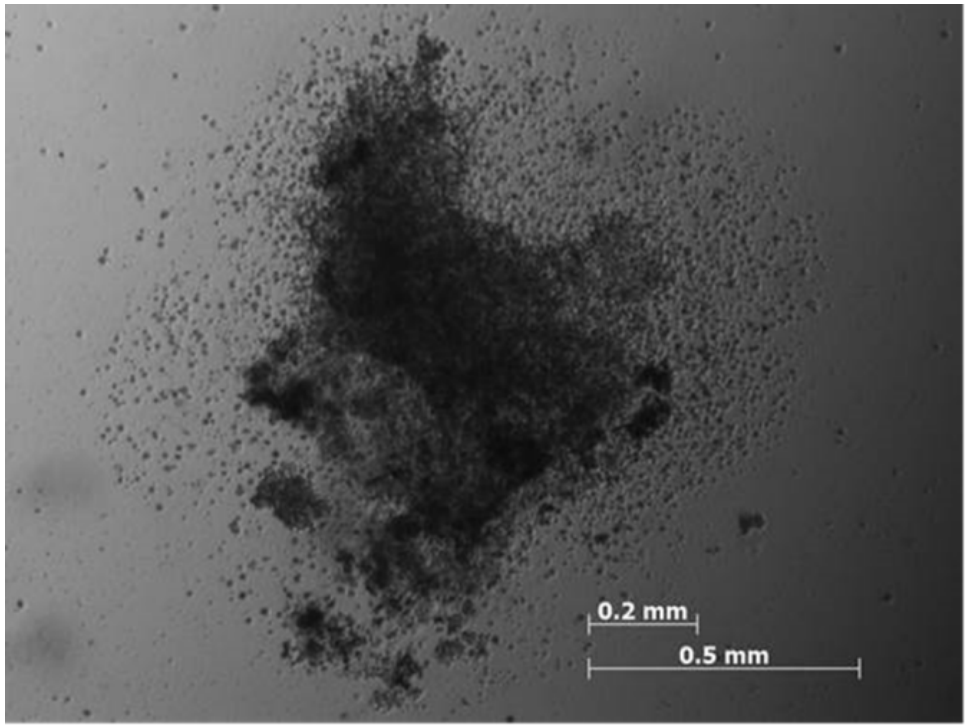
Current methods are short-term primary cell culture lasting 3-6 population doublings

- No ability to engineer cells for long-term nacre production

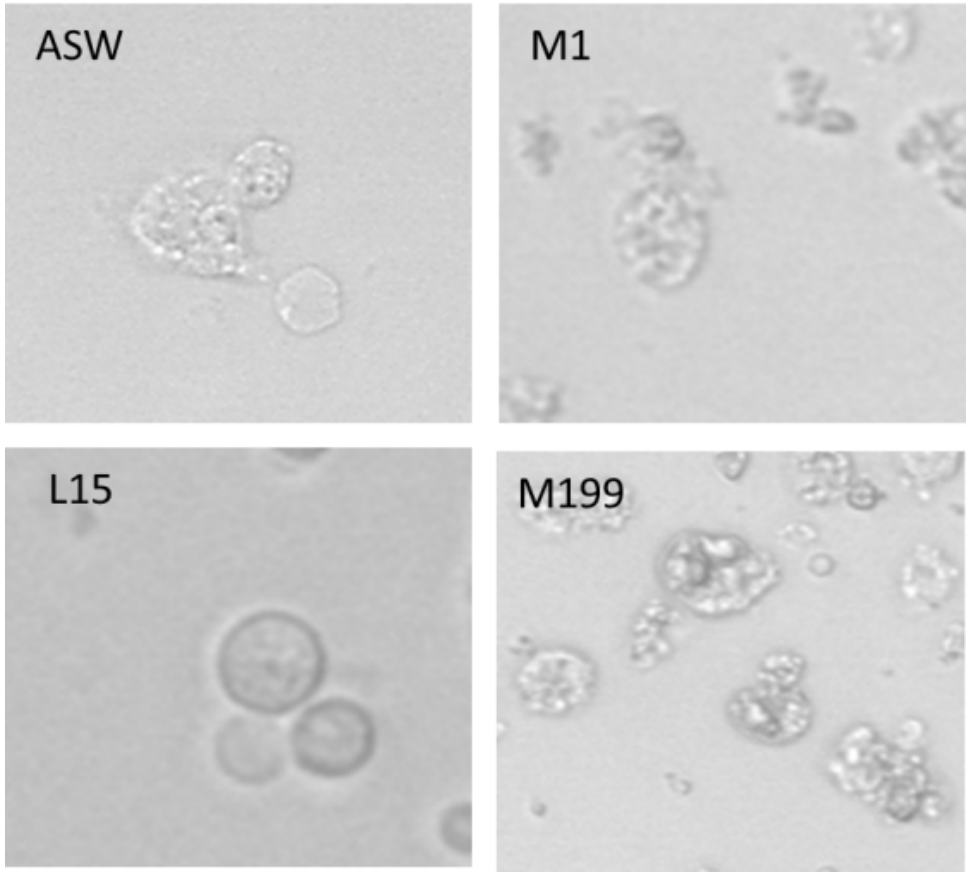
Long-term goal of creating cell lines and tools for genetic transformation



# Primary culture after dissection




Mantle strip with cells migrating out




Media choice affect cell type and culture

- ASW, M1 and M199 grow irregular cells
- L15 grow round cells
- M199 results in high levels of extracellular protein

**Cells survive long term- 3 months, but stop proliferating after ~5 weeks**

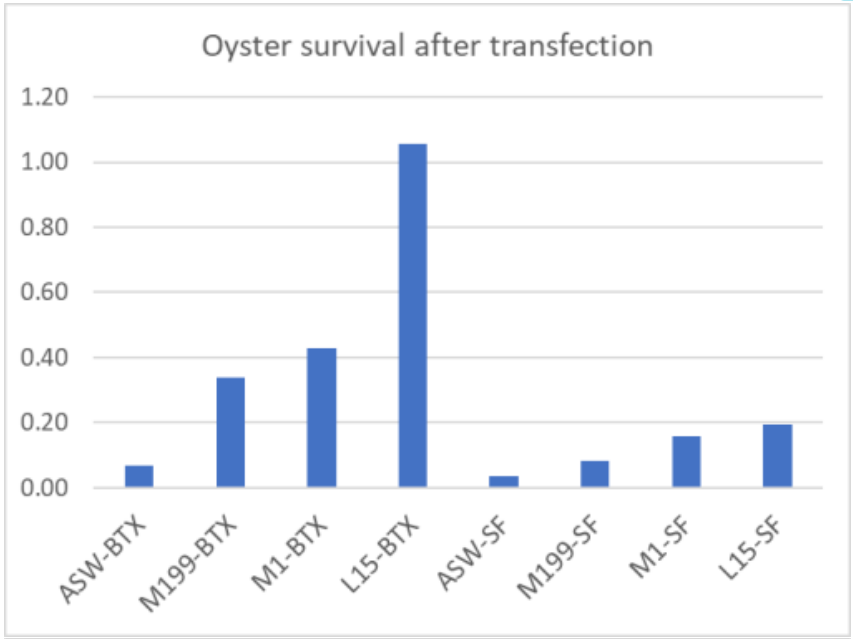
# Transfection method effect on cells

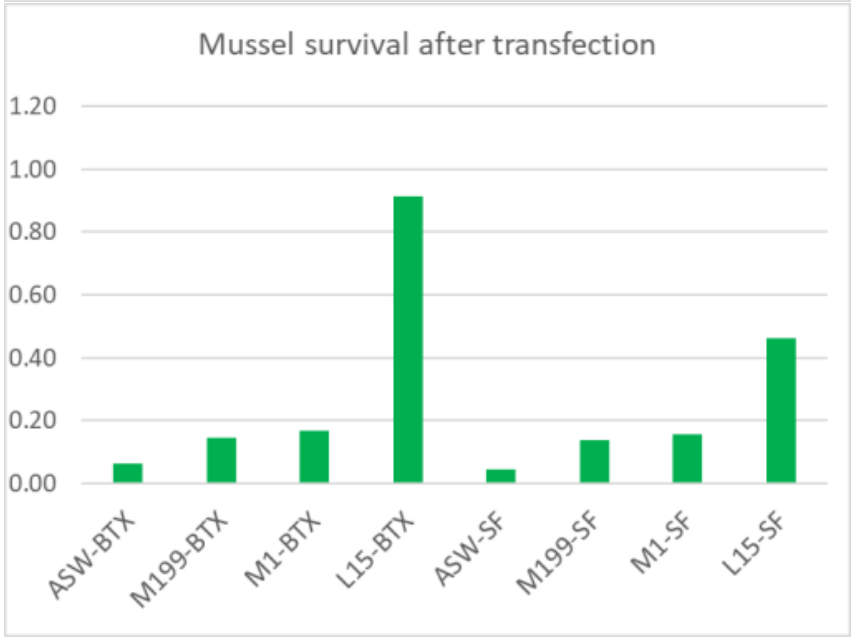
**Three methods examined**

1) Nucleofection-
   - Lonza 4D Nucleofector
2) Electroporation-
   - BTX 830 system from Harvard Apparatus
3) Chemical transfection-
   - Superfect Transfection reagent from Qiagen

Electroporation and chemical transfection demonstrated transfection and were examined in more detail.

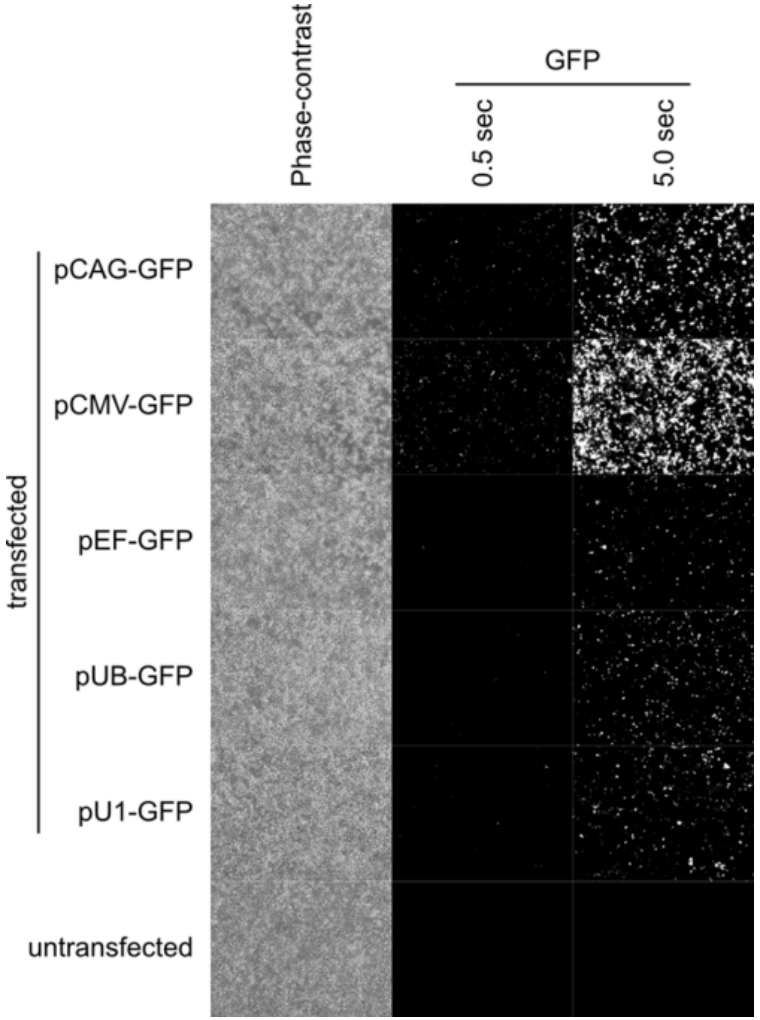
Electroporation resulted in increased cell survival and L15 media resulted in the highest survival rates. ASW media had poor cell survival



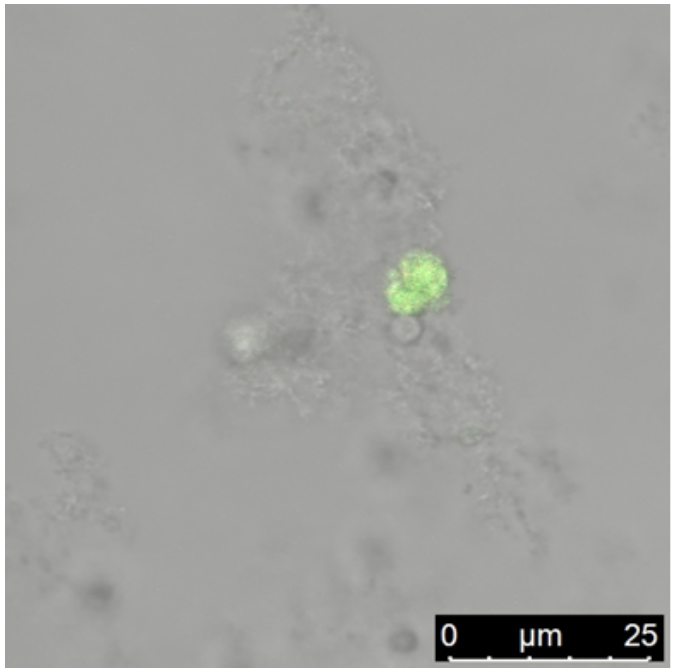



# Transfection of mollusks with plasmids

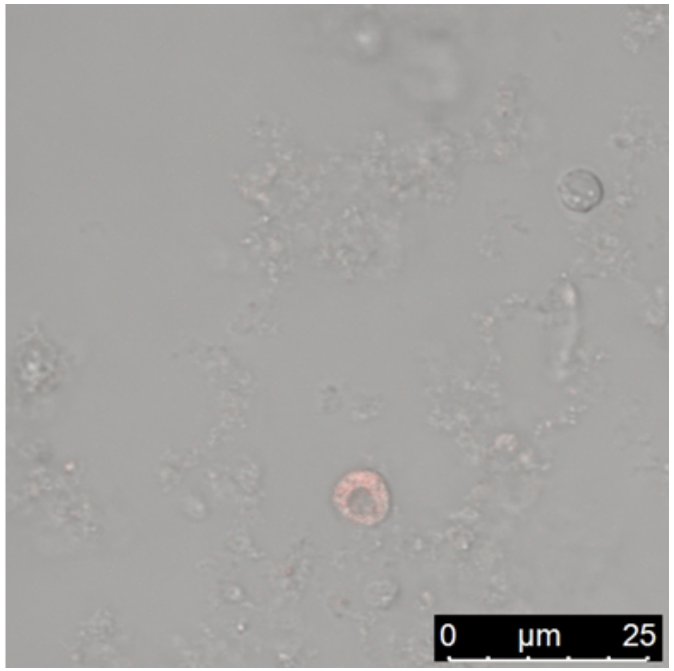
No plasmids with mollusk specific promoters are available
5 mammalian promoters were tested




All 5 plasmids work in mammalian cells




CMV promoter with GFP




CAG promoter with RFP

The CMV and CAG promoters work in both mollusks, but the transfection efficacy is low

Mollusk specific promoters may be necessary for increased efficacy and expression

# Identification of putative mollusk promoters and creation of mollusk specific plasmids

Publicly available mollusk genomes were examined for homologs of highly expressed genes

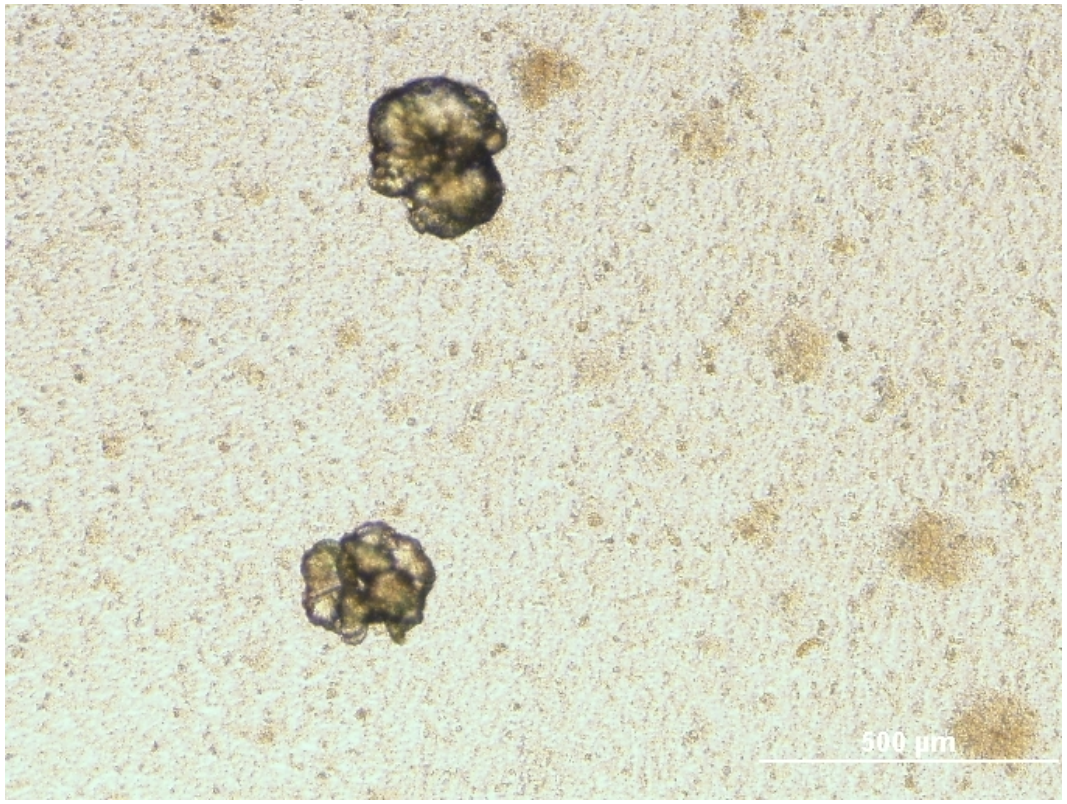
- Elongation factor (EF)
- Ubiquitin (UB)

Putative promoters in the upstream regions were then cloned into plasmids

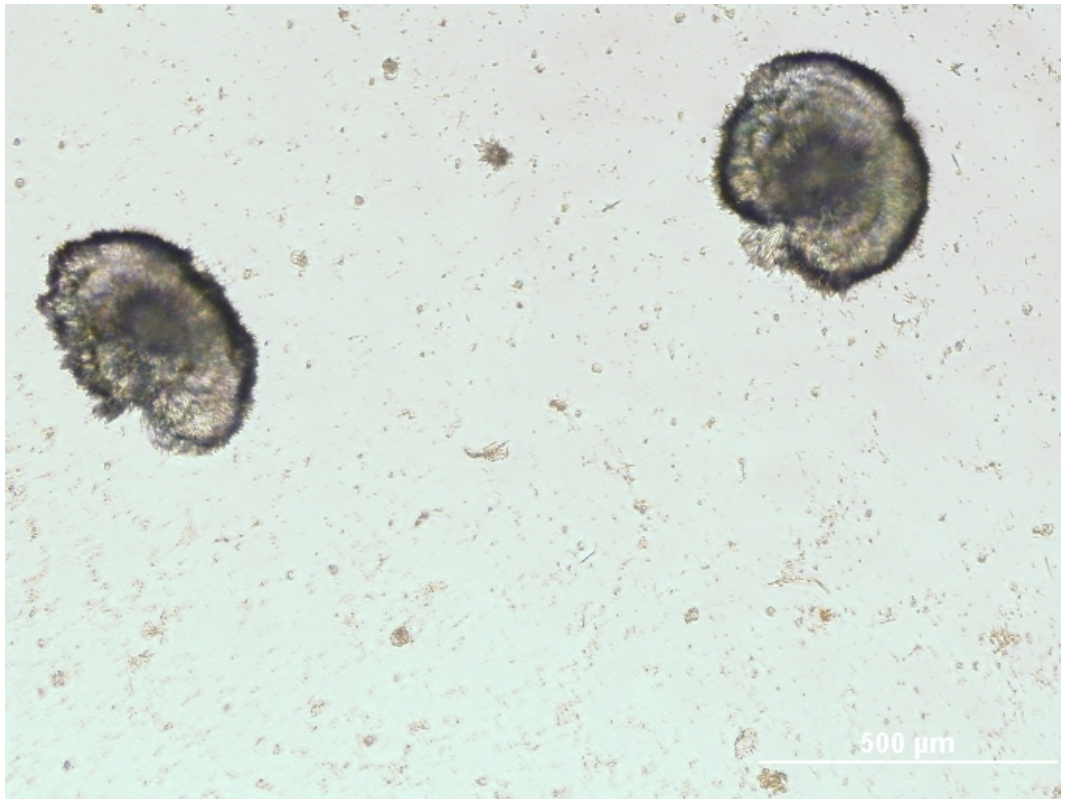
| Plasmid name | Description |
|---|---|
| pcgiEF-GFP | *Crassostrea gigas* EF-1α 2,304 bp fragment upstream of exon 1, intron 1, 30 bp exon 2 upstream translation ATG |
| pshort_cgiEF_GFP | *Crassostrea gigas* EF-1α 342 bp fragment upstream of exon 1, intron 1, 30 bp exon 2 upstream translation ATG |
| pcviEF-GFP | *Crassostrea virginica* EF-1α 2,000 bp fragment upstream of exon 1, intron 1, 29 bp exon 2 upstream translation ATG |
| pshort_cviEF_GFP | *Crassostrea virginica* EF-1α 491 bp fragment upstream of exon 1, intron 1, 29 bp exon 2 upstream translation ATG |
| pmedEF-GFP | *Mytilus edulis* EF-1α 3,500 bp fragment upstream translation ATG |
| pshort_medEF_GFP | *Mytilus edulis* EF-1α 1,500 bp fragment upstream translation ATG |
| pcviUB-GFP | *Crassostrea virginica* UBC 2,806 bp fragment upstream of exon 1, intron 1, 30 bp exon 2 upstream translation ATG |
| pshort_cviUB_GFP | *Crassostrea virginica* UBC 806 bp fragment upstream of exon 1, intron 1, 30 bp exon 2 upstream translation ATG |
| pmedUB-GFP | *Mytilus edulis* UBC 3,500 bp fragment upstream of translation ATG |
| pshort_medUB_GFP | *Mytilus edulis* UBC 1,500 bp fragment upstream of translation ATG |

# Crystal formation from mollusk cultures

Oyster in M199 media




Mussel in M1 media




Overtime crystals were formed by the mussel cells
Formation was possibly cell type dependent and media dependent

- Without growth factors, no crystals formed
- The round cells selected by L15 media did not form crystals

# Nacre next steps and ongoing work

Genetic engineering

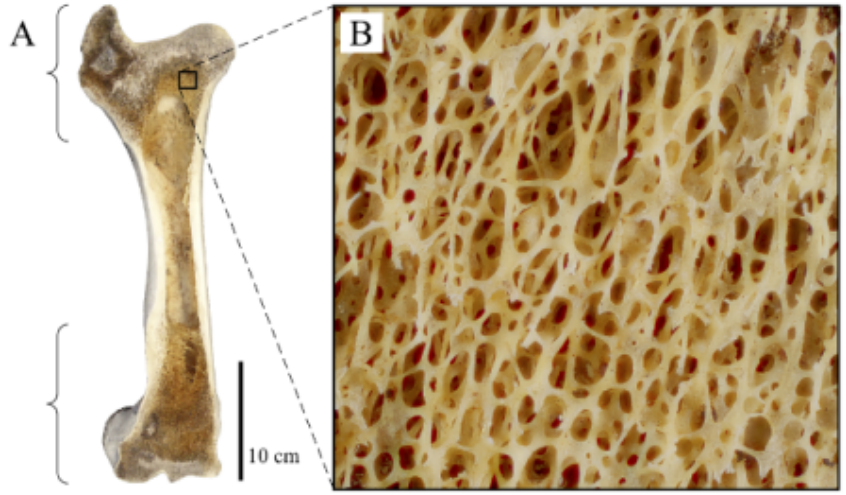
Test expression potential of the new mollusk promoters

Create plasmids that target nacre production genes

Materials characterization

Examine the structure of the crystals formed by both mussel and oysters

# Focus on bone




Bishop et. al. *PeerJ*. 2018. 6:e5778.

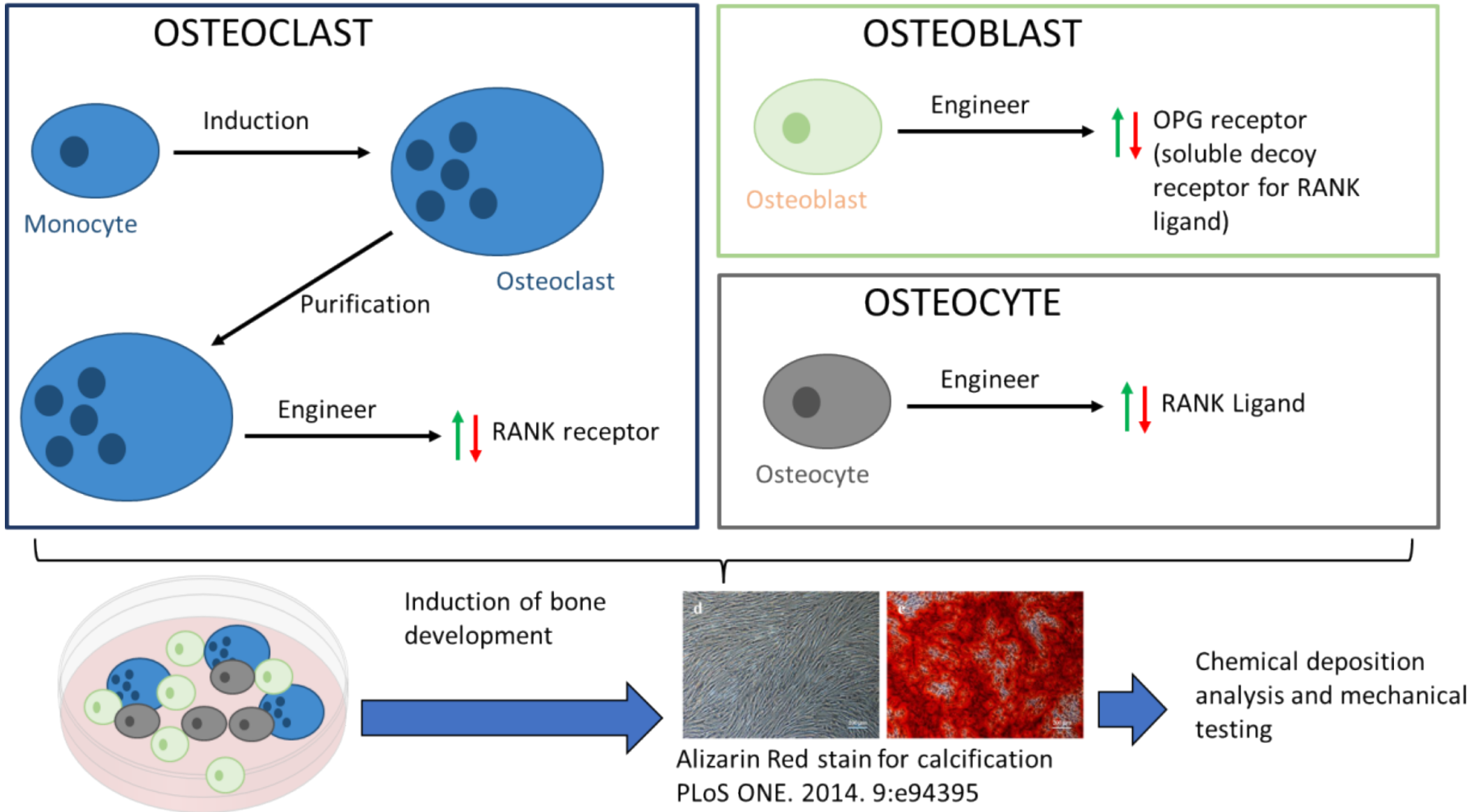
**Bone:**

- Lightweight
- Withstands high impact from many directions & cyclic fatigue
- Moderate mechanical stress increases strength
- Continuous regeneration
- Sense & repair damage

These functions are a product of a complex 3 cell type system and all three types are needed to replicate these features

- Osteoblasts create bone
- Osteoclasts destroy bone
- Osteocytes live in bone and sense damage and stress, recruit osteoblasts and osteoclasts for remodeling

# Long term goal: 3 cell model development



# Development of a functional assay for Osteoclast activity

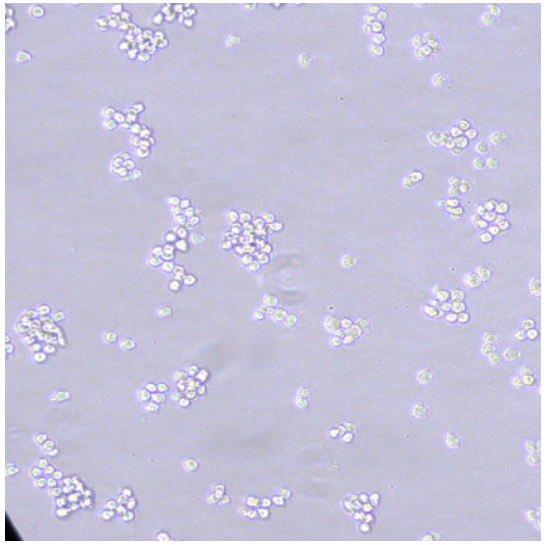
## Induced osteoclasts destroy artificial bone substrate

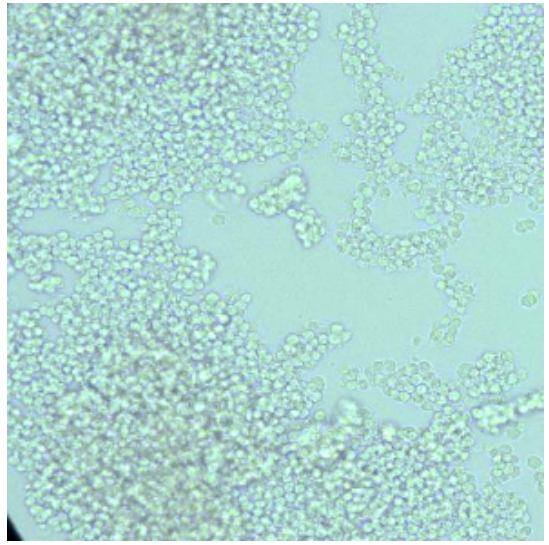
Day 1

Day 7

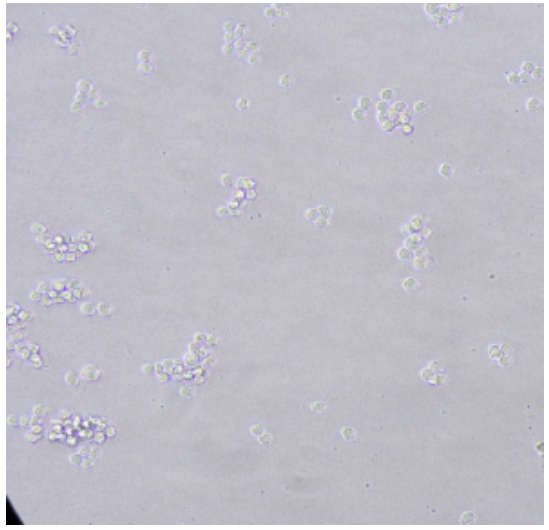
Day 7 bone substrate: stained with silver nitrate

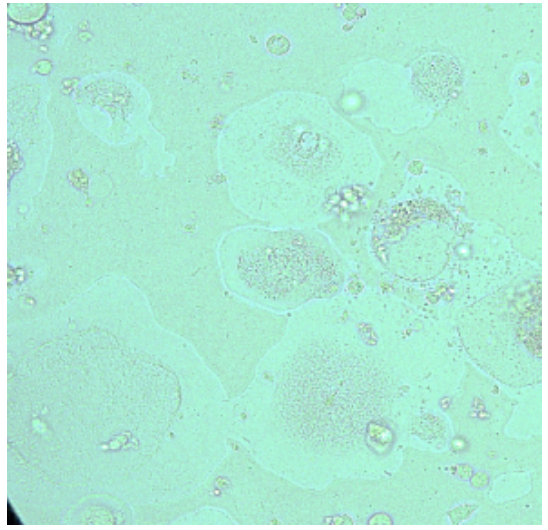
Control: RAW cells no induction

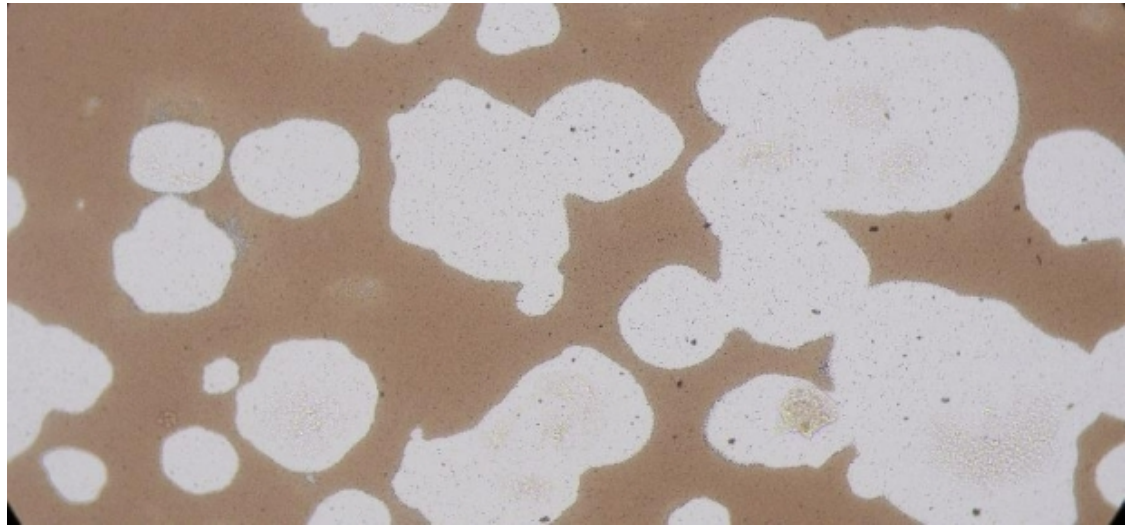




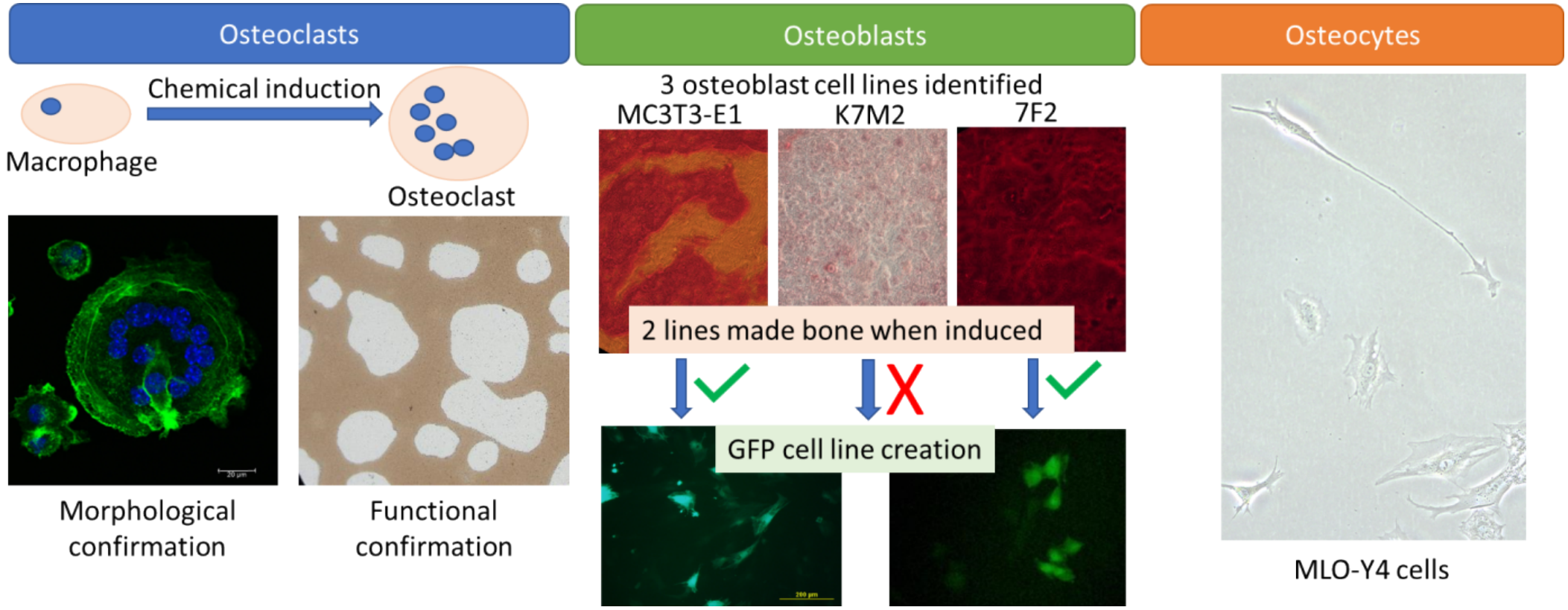
RANKL induced RAW cells







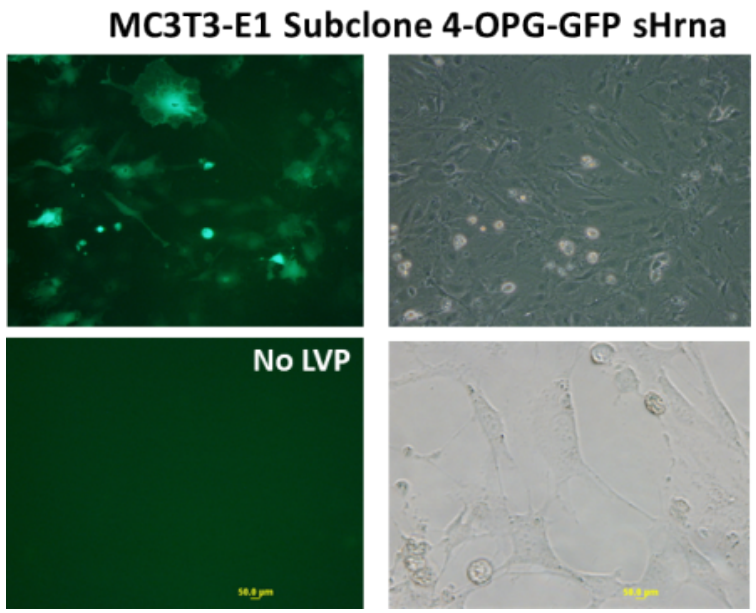
# Progress developing a 3 cell type model with bone induction

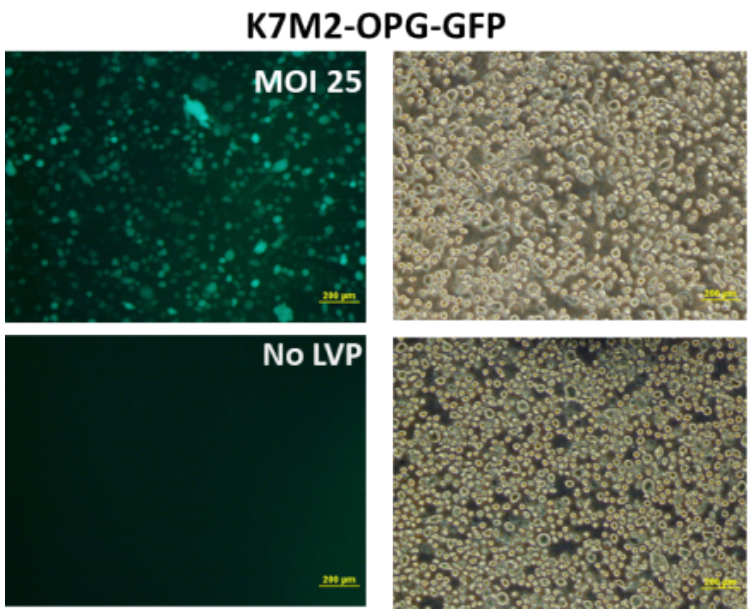


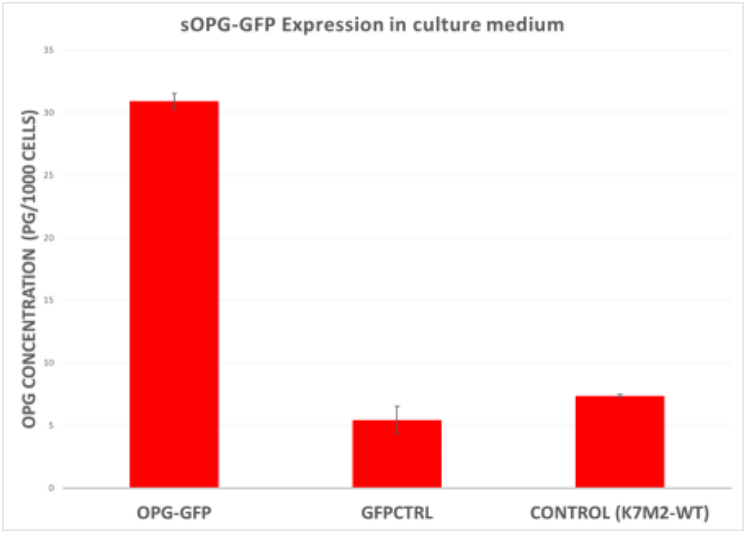

To facilitate 3D visualization of all three cell lines, osteocytes are being engineered to express RFP

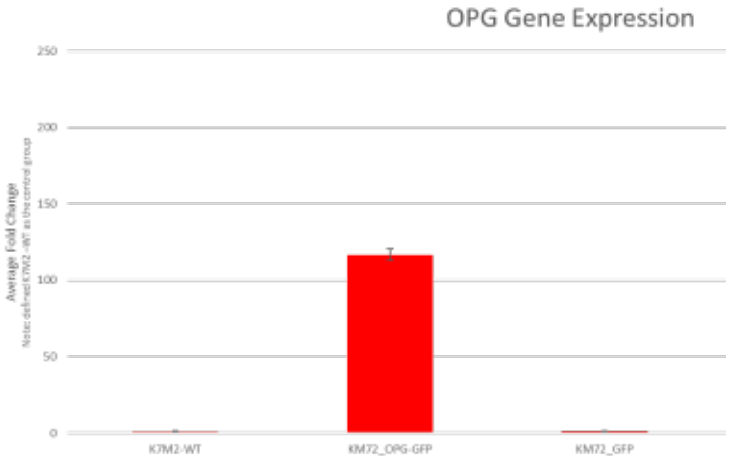
# SynBio-based control of OPG expression

Created and confirmed modified cell lines capable of increased OPG production in K7M2 osteoblast cell line and decreased OPG production in MC3T3-E1,subclone 4 osteoblast lines.



# *In vitro* bone development

**Successes**

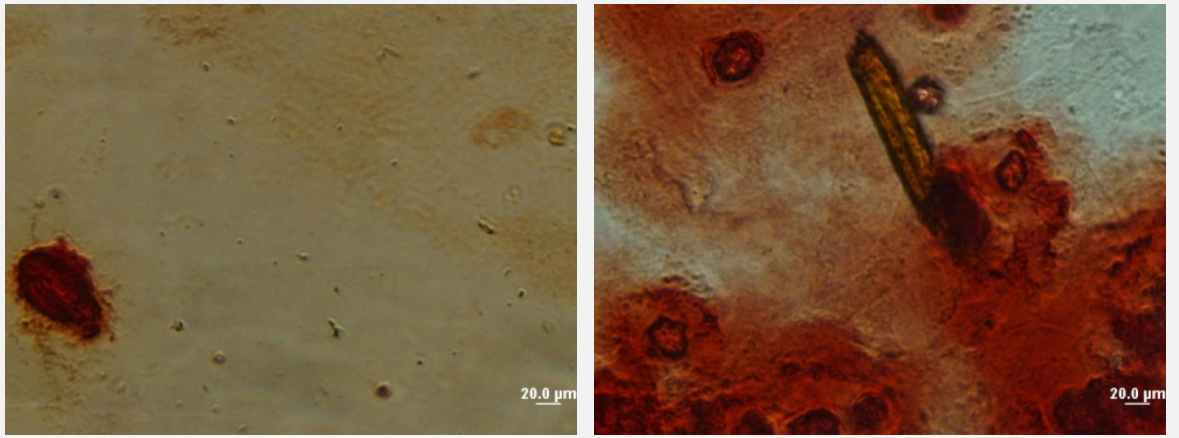
We have successfully developed a protocol that induces bone formation in 2 of the 3 cell lines on plastic and 1 of the 3 cell lines on glass

Bone formation on plastic covers the entire surface, Bone thickness is 20µm using current protocols

## Induced osteoblast cultures stained with Alizarin Red for bone formation

Glass substrate

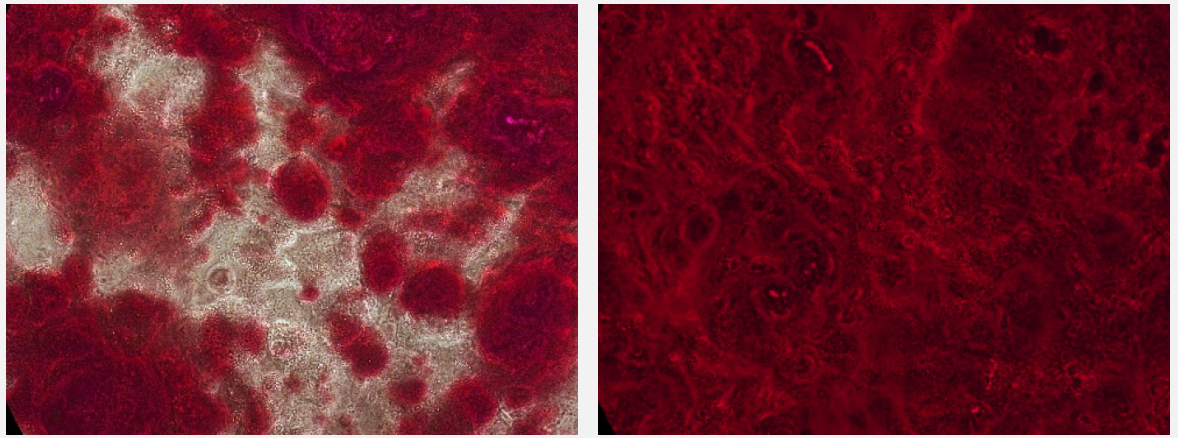
Plastic substrate




First round of bone induction

Glass substrate

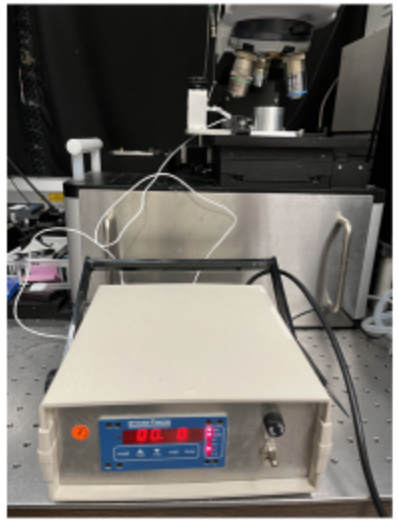
Plastic substrate



Induction with current protocol

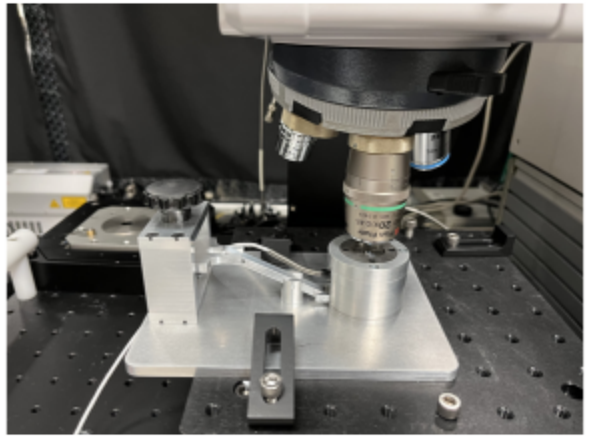
# Measuring Stress in *in vitro* grown bone

**Pressure Gauge Display**

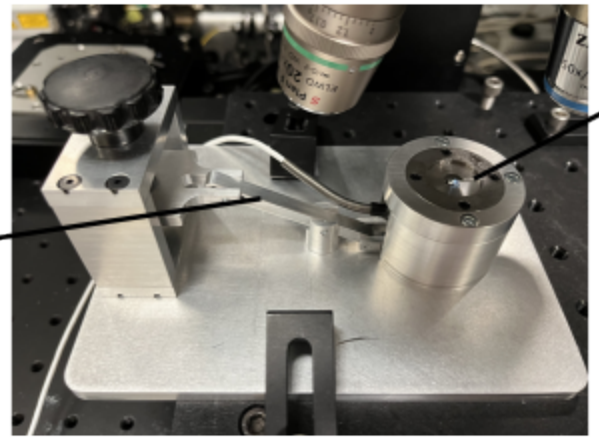


*Applied force is monitored by pressure gauge and digital readout.*

**Flexure Stage Under Raman Scope**



**Raman Scope**
*Raman measurements can be performed as a function of applied stress*



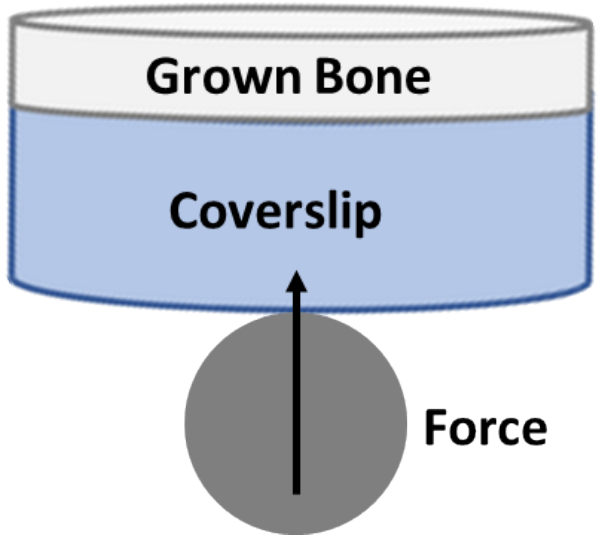
**Fixed Coverslip**
*Steel ball raises and applies equibiaxial flexure to coverslip.*

**Lever**
*Raises steel ball underneath fixed coverslip to apply stress.*

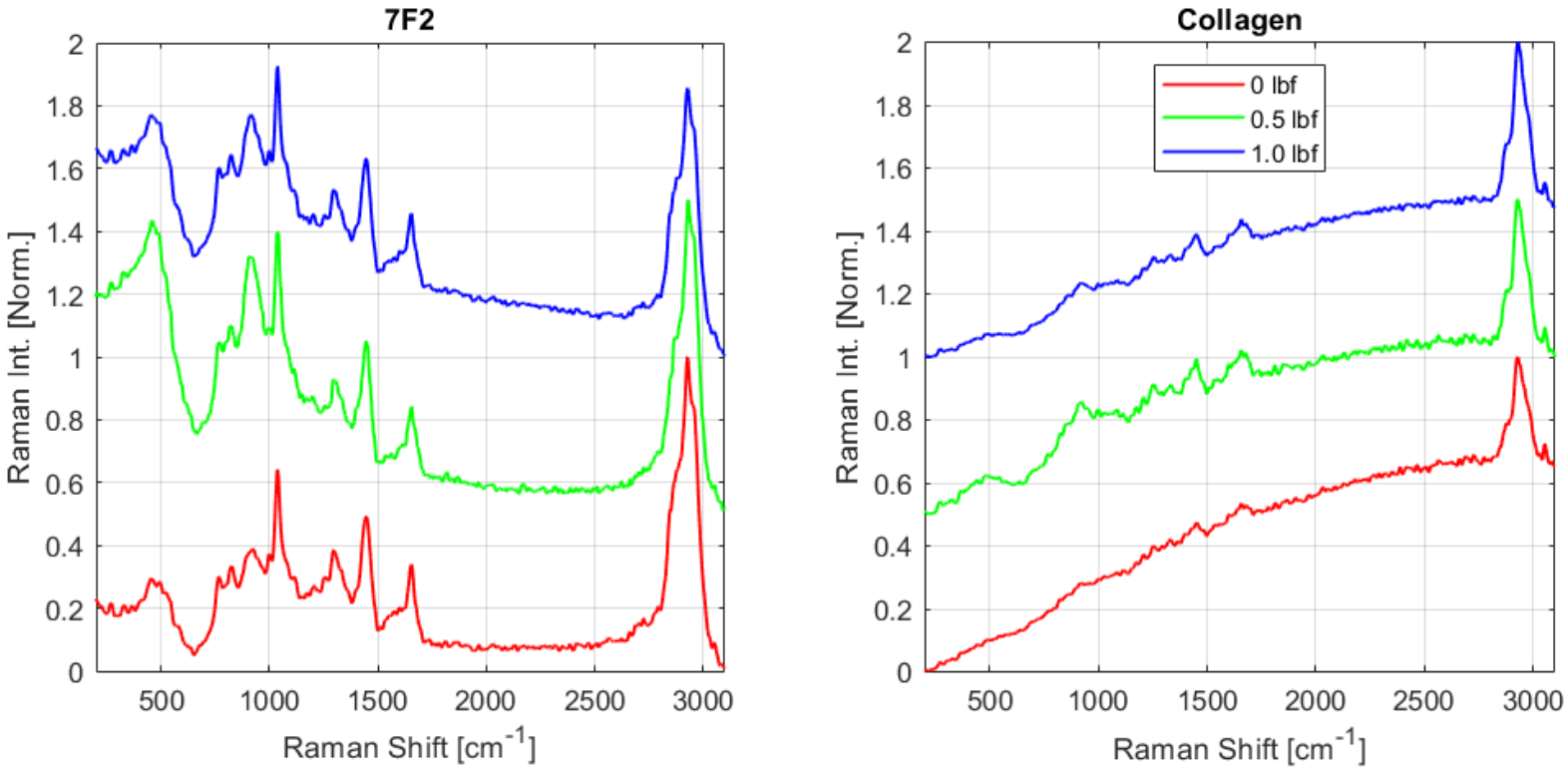
Raman shifts are indicative of stress and correlate with bone strength

**Challenge:** In vitro grown bone is grown on thin, circular coverslips.
**Solution:** Equibiaxial flexure apparatus.

**First Attempt:** Coverslips likely too fragile. Need a more flexible substrate.

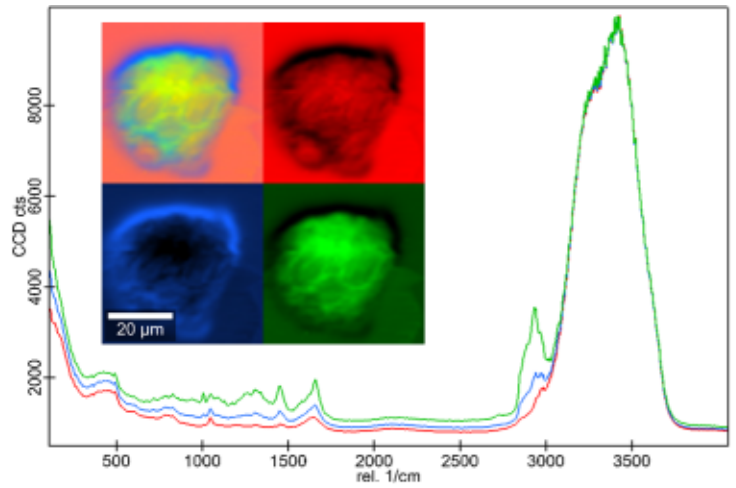


# Preliminary Results Under Applied Stress




High enough stresses were not reach before breaking the coverslip substrate. No notable peak shifting in spectra up to 1 lbf.

# Classification of Bone Cells via Raman Spectroscopy



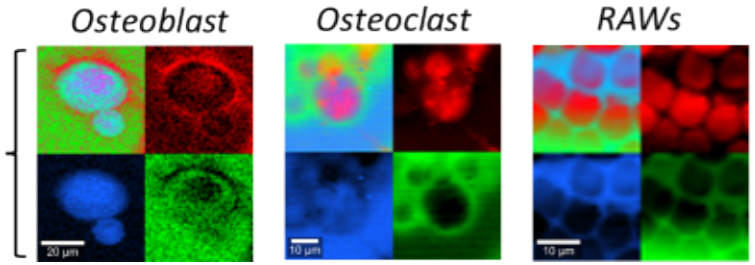

**Example Hyperspectral Raman Image:**

Osteoclast cell imaged using a 488 nm Raman laser.

Unique spectral components differentiated by k-means clustering.

---

**Example images taken at 785 nm**




## Path Towards Publication

- Collect Raman images of osteoclast, osteoblast, and RAWs with 785, 532, and 488 nm Raman lasers. ✓
- Use MCR and/or k-means clustering to parse unique spectral components. ✓
- Compile data into library. Explore regression methods for classification of unknown mixtures. ✓
- Measure unknown mixture and/or generate synthetic mixture image.
- Test classification method for ability to distinguish cell types in an unknown mixture.

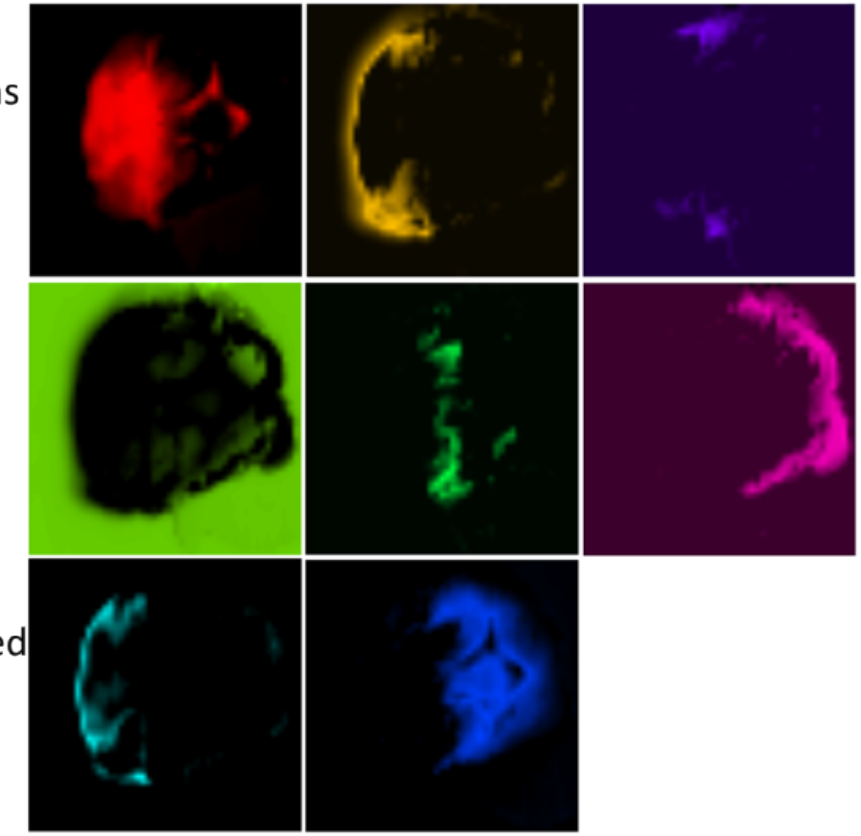
## Classification Progress

- Code written to perform k-means clustering on cell Raman library
- Code written to fit components pixel-by-pixel to maps → spatial distribution of components.

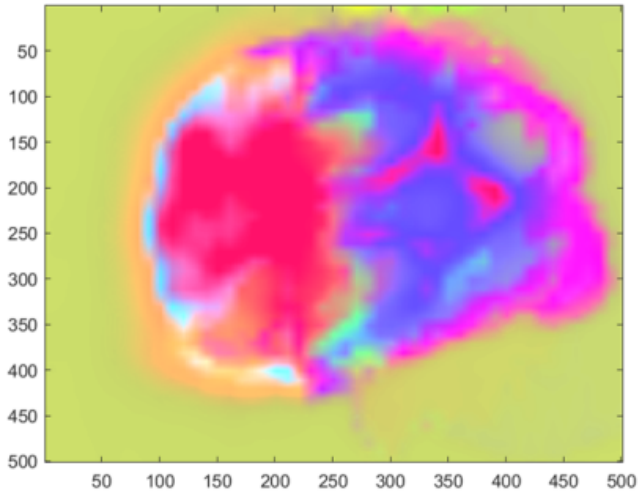
**Next Steps:**

- Assign component spectra to known vibrational features.
- Fit “unknown” cell to all extracted components. The weight of components should indicate the cell type.

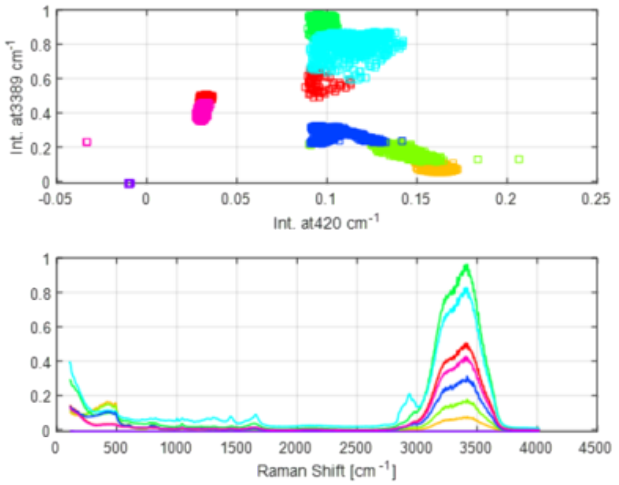
## K-Components



## Overlay Map




## Spectral Components and Clustering

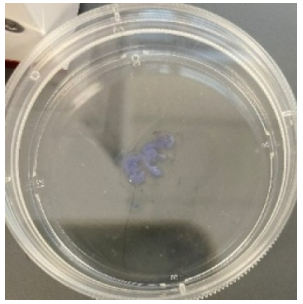


# 3D printing bone scaffolds

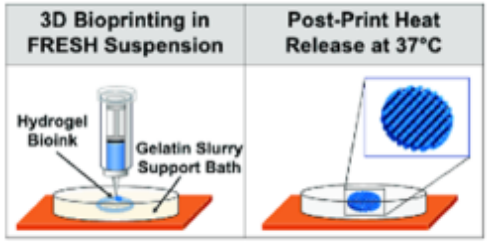
Goal:  3D bioprinting of 3 different scaffolds to promote structure specific bone growth.

Progress: 3 different bioink formulations, 3 printing configurations have been tested, toxicity tests complete

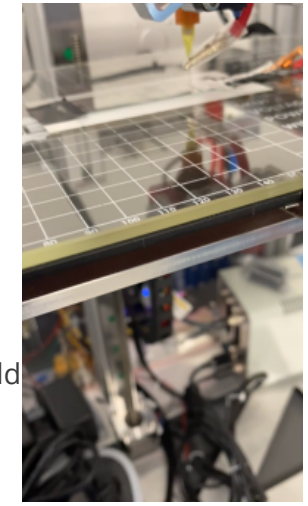
1. Collagen Type I, Rat Tail crosslinked via Genepin
    1. Control
    2. + Hydroxyapatite nanoparticles
    3. + β-TCP microparticles
2. Methacrylated Collagen Type I, Bovine Hide crosslinked via Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP)
    1. Control
    2. Bioactive glass microparticles
3. Collagen Type I, Bovine Tendon solubilized via 40% Ascetic Acid
    1. Control
    2. + Hydroxyapatite nanoparticles



Lack of structure in Genepin-based bioinks via direct-write printing




FRESH immersion printing schematic



Video of near-field electrospinning (NFE) process

# Bone next steps and ongoing work

Genetic engineering

Engineer additional osteoblast lines for OPG and RANKL

Mechanical characteristics

Create new bone samples and test mechanical indentation

3 cell model

Take the GFP engineered osteoblasts, RFP engineered osteocytes and the osteoclasts and create a mineralized model.  3D image via confocal microscopy

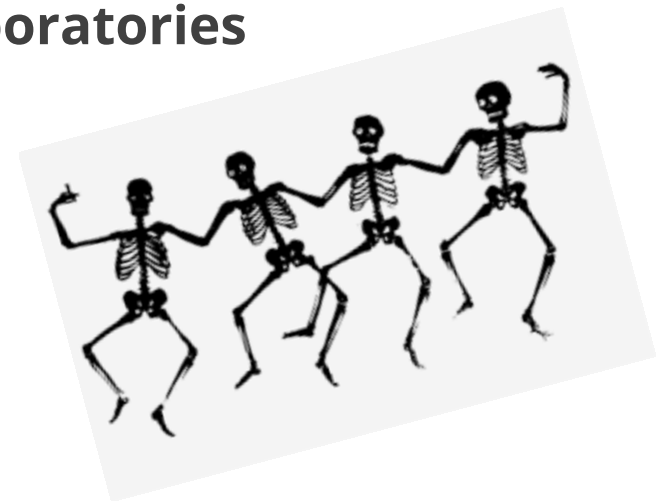
3D scaffold

Mineralization via osteoclasts is underway, characterization to follow

# Thank you!

## Skeleton Crew

**Sandia National Laboratories**
Jerilyn Timlin
Dulce Hayes
Zak Pointkowski
Steve Meserole
Anthony McDonald
Kevin Strong
Wittney Mays
Kalista Pattison



**UNM**
Christina Salas and team

## Team Nacre

**Sandia National Laboratories**
Joshua Podlevsky
Andreana Nourie
Emmarie Ryan
Kalista Pattison
Elizabeth Nail
Anastasia Hendricks
Braden Rue