Combinatorial Cassettes: A Systematic Approach for Evaluating Osteogenic Constructs In Vivo

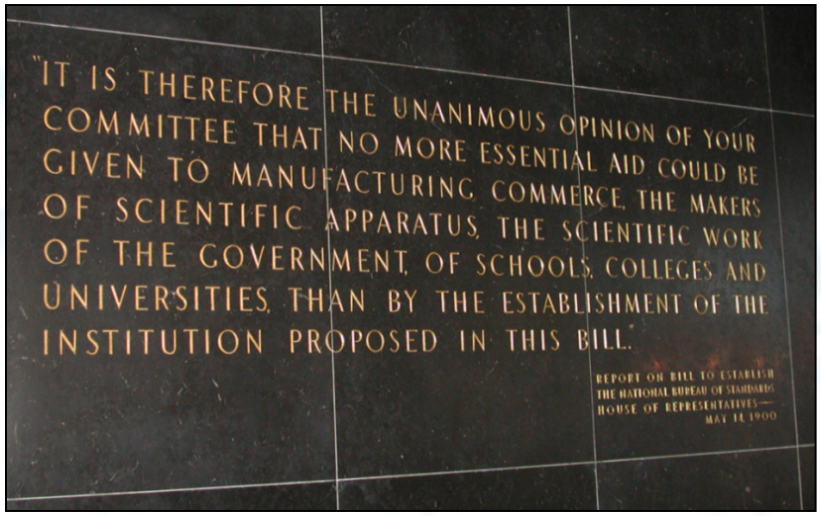
Carl G. Simon, Jr., Ph.D.
Biologist
Gaithersburg, MD

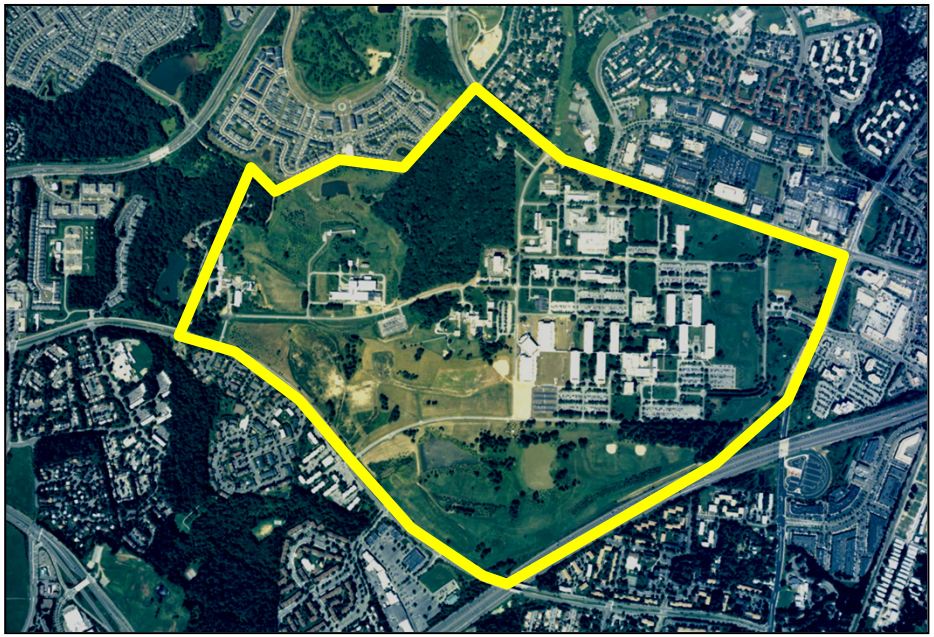
National Institute of Standards and Technology
United States Department of Commerce

Materials Measurement Laboratory • Biosystems & Biomaterials Division • Biomaterials Group

NIST

- **Mission:** to promote U.S. innovation and industrial competitiveness by advancing measurement science, standards, and technology in ways that enhance economic security and improve our quality of life
- Established by congress in 1901 as the nation's measurement lab
- 5000 staff, 3000 PhDs, 4 Nobel Laureates since 1997, 8 current National Academy members
- 2016 budget $964M





Acknowledgements

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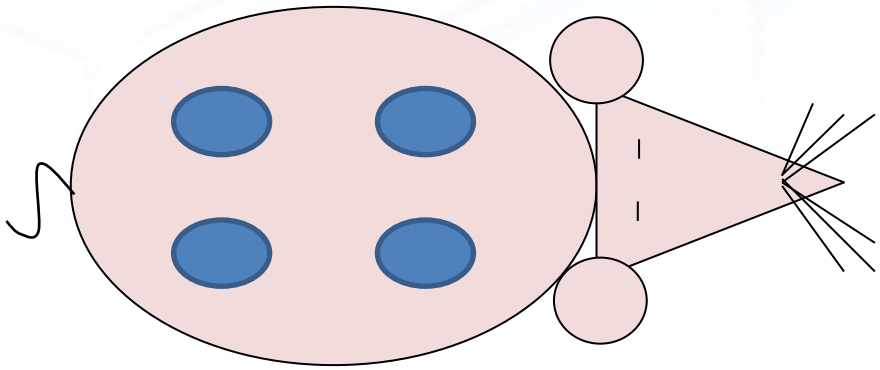
Bonfin, Tina Kilts, Li Li, Marian Young

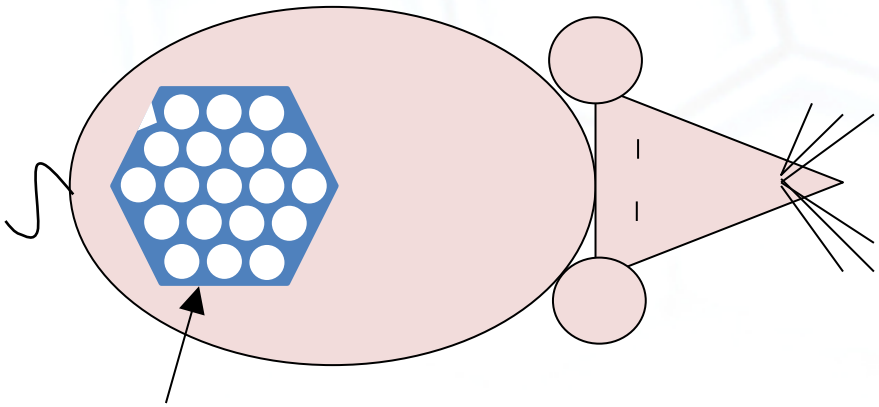
Aim: Increase Number of Osteogenic Formulations that Can Be Tested in a Mouse

Traditional Approach (Non-Combi)



Traditional, non-combi approach is 4 implants per mouse

Combinatorial Cassettes (Combi-Cassette)



Combi-cassette holds up to 19 implants

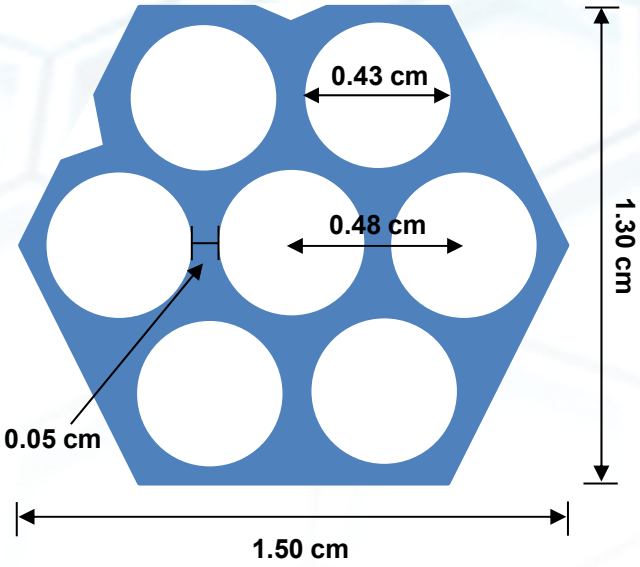
- 7-Well Cassette: 7/4 = 1.75-fold increase
- 19-Well Cassette: 19/4 = 4.75-fold increase

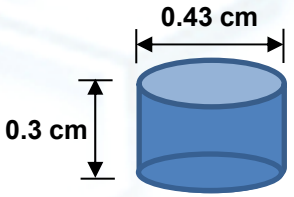
Why Increase Throughput In Animal Testing?

- Animal tests are the most biologically relevant platform for assessing tissue regeneration (besides human clinical trial)
- Ethical reasons: use fewer animals, or get more data from each mouse
- Animal testing may be slow: ≈6 months per data point in our case
    - Sample preparation + cell expansion (3 wks) + surgery + implantation time (2 mos) + histological processing (2 mos) + scoring + μCT + PCR + data analysis = 6 mos
- Animal testing can be highly variable: more data = stronger conclusions
    - Experiment worked 3 times out of 7 tries
        - Issues: phenotypic drift, cell expansion (serum), cell seeding density onto scaffolds, particle size of scaffold, need one mouse per cage, staples came undone, cell source (donor, bone marrow aspirate vs. surgical waste bone fragments)

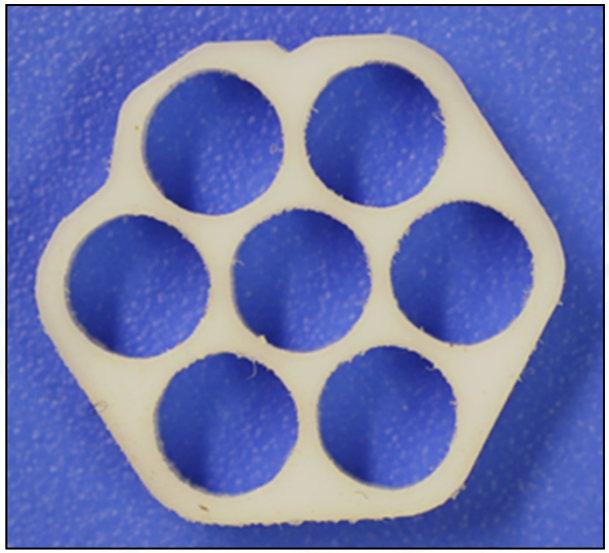
Lasercut Teflon (PTFE)

7-Well Combi-Cassette

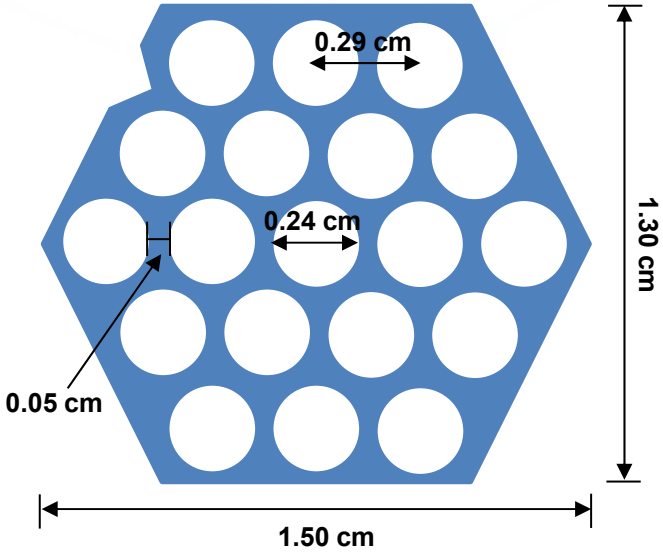


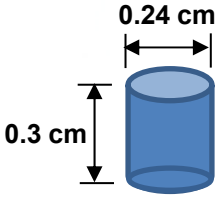


Well Volume = 43.5 µL

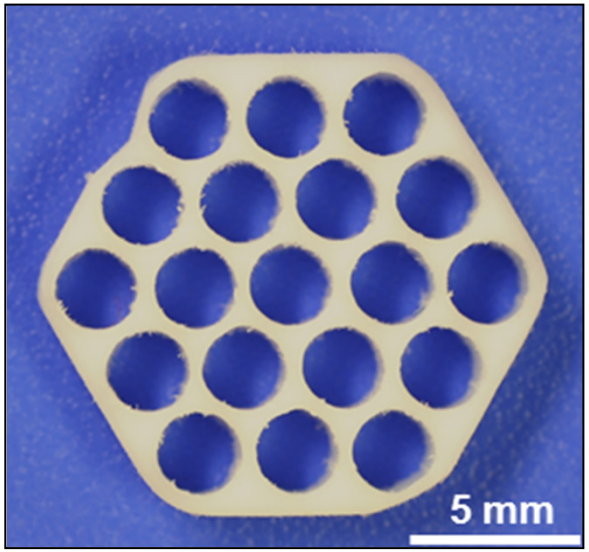


19-Well Combi-Cassette





Well Volume = 13.5 µL



Goal: Validate Combi-Cassette Against Traditional Non-Combi Approach

Test 2 Types of Constructs:

- **Cell-Based (8 Weeks)**
  - Primary Human Bone Marrow Stromal Cells
  - HA/TCP Particles
  - Fibrin Gel (to hold it together, improves handling)
- **Growth Factor-Based (8 Weeks)**
  - rhBMP-2
  - Gelatin Sponge

Only analyzed data from experiments where the "traditional non-combi" implants yielded good bone formation

Primary Human Bone Marrow Stromal Cells (hBMSCs)

1997 paper with 500 citations

0041-1337/97/6308-1059$03.00/0
TRANSPLANTATION

Vol. 63, 1059–1069, No. 8, April 27, 1997
Printed in U.S.A.

BONE FORMATION IN VIVO: COMPARISON OF OSTEOGENESIS BY TRANSPLANTED MOUSE AND HUMAN MARROW STROMAL FIBROBLASTS

PAUL H. KREBSBACH,[1,2] SERGEI A. KUZNETSOV,[3] KAZUHITO SATOMURA,[3] ROBERT V. B. EMMONS,[4] DAVID W. ROWE,[5] AND PAMELA GEHRON ROBEY[3,6]

- Fibroblastic cell preparation from marrow that adhere to plastic and are osteogenic, adipogenic & chondrogenic & may form hematopoietic marrow organs in vivo
- Used in 100s of clinical trials
- **Mouse Sub-Cutaneous Implantation Model for Heterotopic (Ectopic) Osteogenesis**

Controls that did not form bone:

- Mouse spleen fibroblasts + gelatin
- Human foreskin fibroblasts + HA/TCP powder
- Human foreskin fibroblasts + HA/TCP powder-bovine collagen strip

Osteogenesis by Bone Marrow Stromal Fibroblasts in Different Transplantation Vehicles

Vehicle	Cells	
	Mouse	Human
Gelatin	21/23	5/28
Polyvinyl Sponge	3/5	0/3
Porous Collagen Matrix	2/2	---
HA/TCP Block	10/10	13/14
Poly(L-Lactic Acid)	---	0/2
Human Demineralized Bone Matrix	---	0/3
Human Demineralized Bone Matrix + Gelatin	---	0/9
Human Demineralized Bone Matrix + Fibrin Clot	---	0/15
HA/TCP Powder	---	13/15
HA/TCP Powder + Gelatin	---	2/4
HA/TCP Powder + Fibrin Clot	---	12/12
HA/TCP Powder + Collagen Gel	---	0/6
HA/TCP Powder + Bovine Coll. Strip	---	20/23

hBMSCs

In vitro tests are easier but are less specific

Denu et al. Fibroblasts & mesenchymal stromal/stem cells are phenotypically indistinguishable. Acta Haemat 136:85-97, 2016

The "2006 Dominici" paper on in vitro differentiation:

International Society for Cellular Therapy ISCT

Cytotherapy (2006) Vol. 8, No. 4, 315–317

Taylor & Francis

POSITION PAPER

Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement

M Dominici[1], K Le Blanc[2], I Mueller[3], I Slaper-Cortenbach[4], FC Marini[5], DS Krause[6], RJ Deans[7], A Keating[8], DJ Prockop[9] and EM Horwitz[10]

Myth of "Universal MSCs"?:

v6, p897-913, 2016

Stem Cell Reports

ISSCR

Article

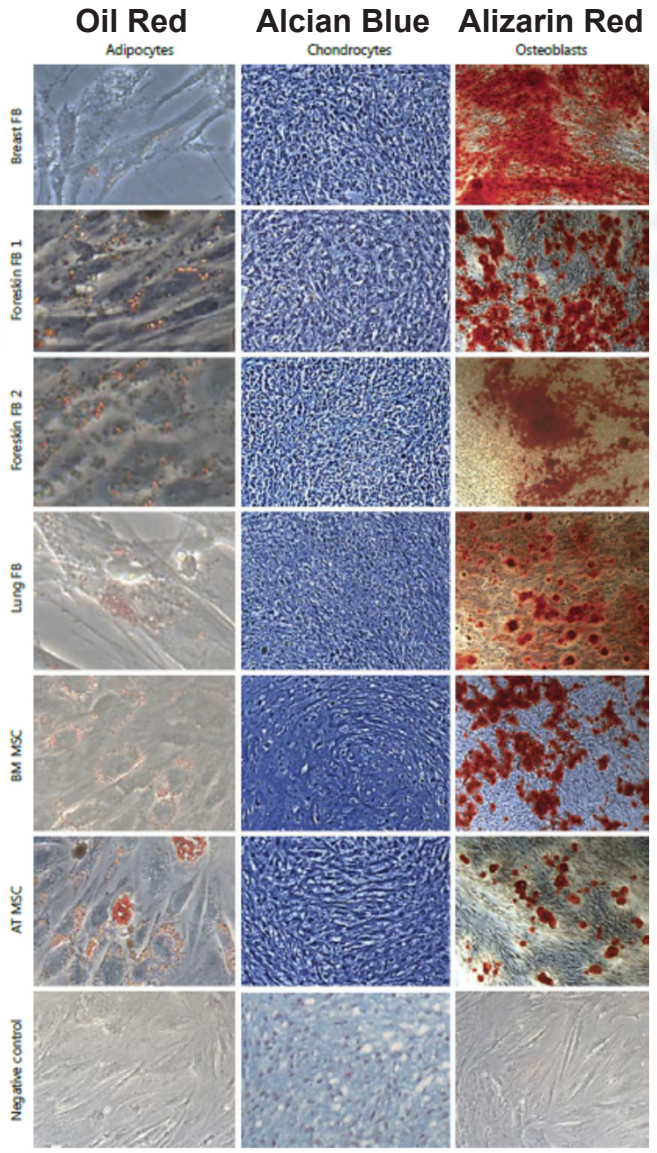
OPEN ACCESS

No Identical "Mesenchymal Stem Cells" at Different Times and Sites: Human Committed Progenitors of Distinct Origin and Differentiation Potential Are Incorporated as Adventitial Cells in Microvessels

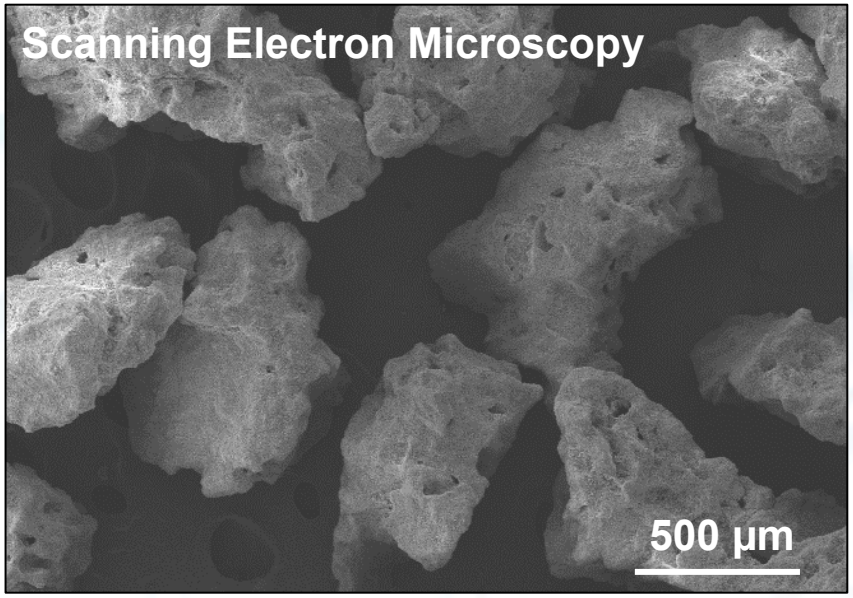
Benedetto Sacchetti,[1] Alessia Funari,[1] Cristina Remoli,[1] Giuseppe Giannicola,[2] Gesine Kogler,[3] Stefanie Liedtke,[3] Giulio Cossu,[4] Marta Serafini,[5] Maurilio Sampaolesi,[6] Enrico Tagliafico,[7] Elena Tenedini,[7] Isabella Saggio,[8] Pamela G. Robey,[9,*] Mara Riminucci,[1,*] and Paolo Bianco[1]

- Marrow MSCs => bone
- Muscle MSCs => muscle (no bone)
- Cord-blood MSCs => cartilage (modest bone)

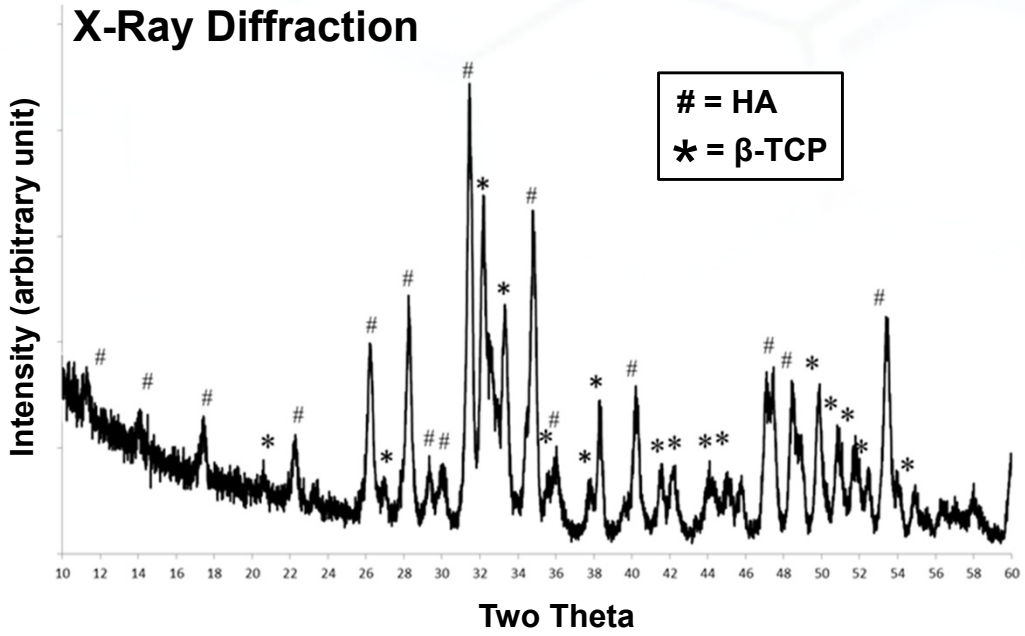
All could guide assembly of functional microvessels in vivo



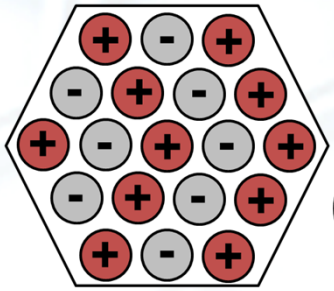
Hydroxyapatite/ β-Tricalcium Phosphate Particles (HA/TCP)



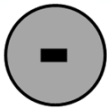
- 65:35 by mass HA/TCP
- 0.5 mm to 1.0 mm nominal particle size
- sterilized 2 h at 200° C
- Zimmer, Inc. (discontinued)



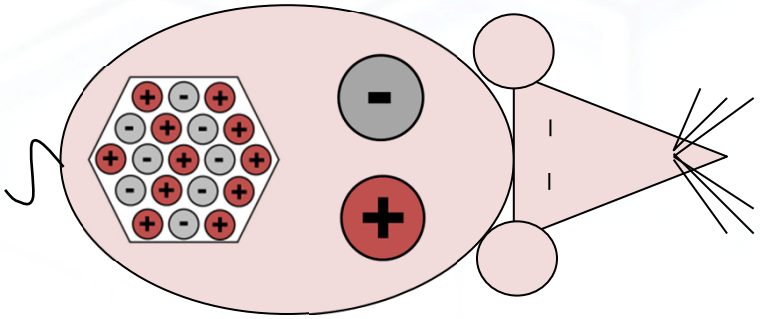
Combinatorial Cassettes (hBMSCs, 8 wks)

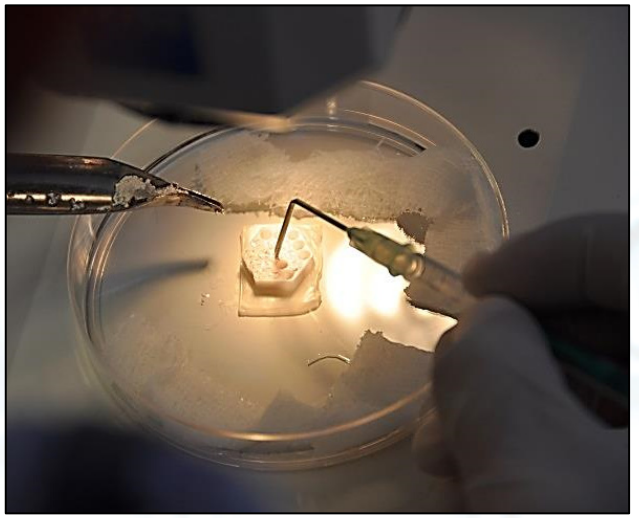


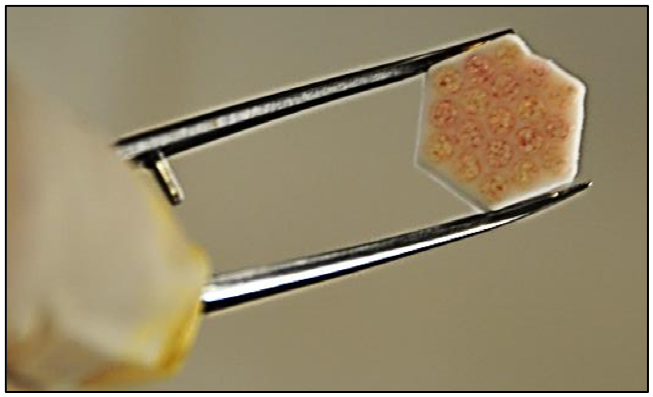
= Positive Control (Osteogenic)
(hBMSCs + HA/TCP + Fibrin)



= Negative Control (Osteogenic)
(HA/TCP + Fibrin)

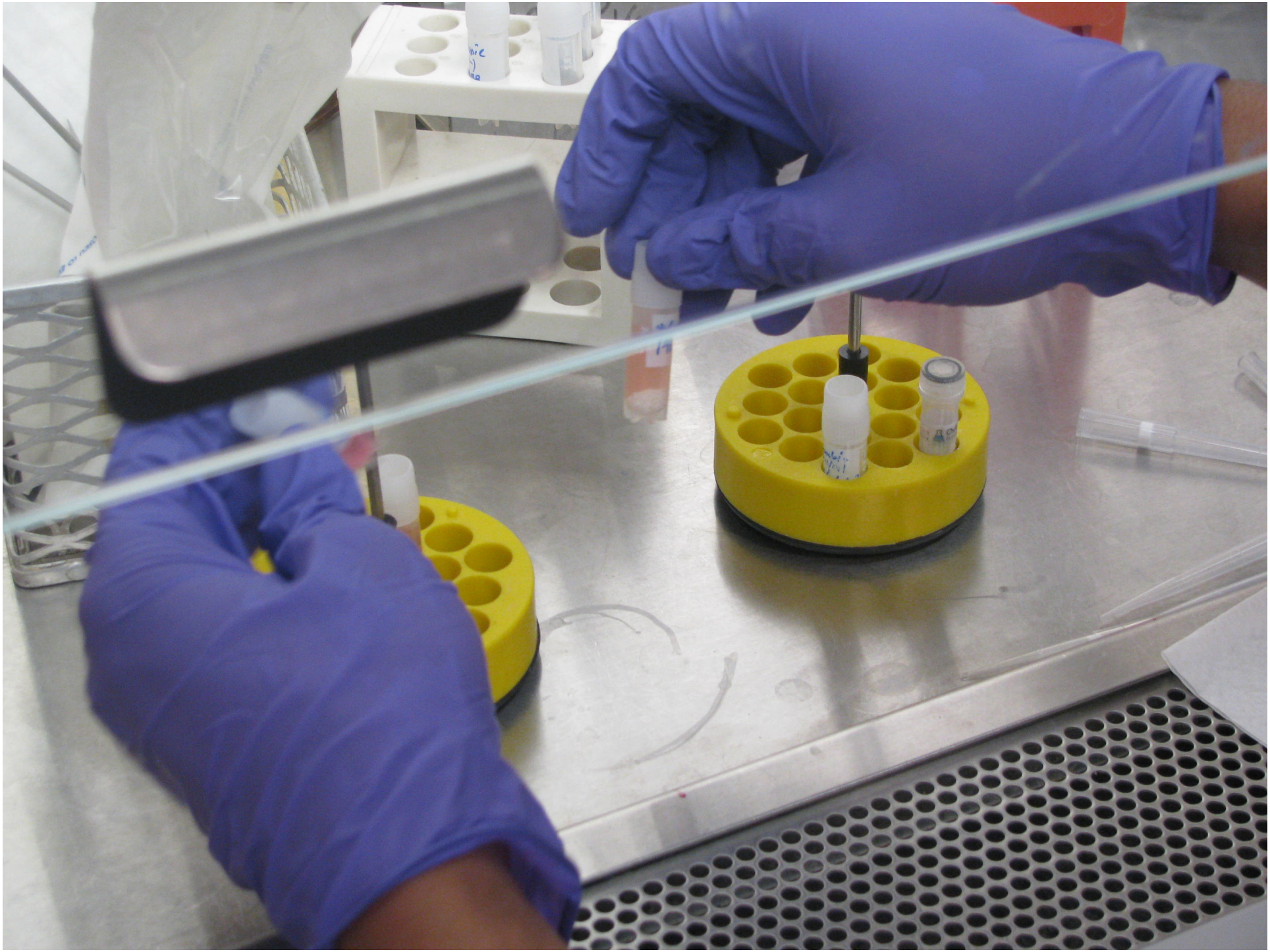




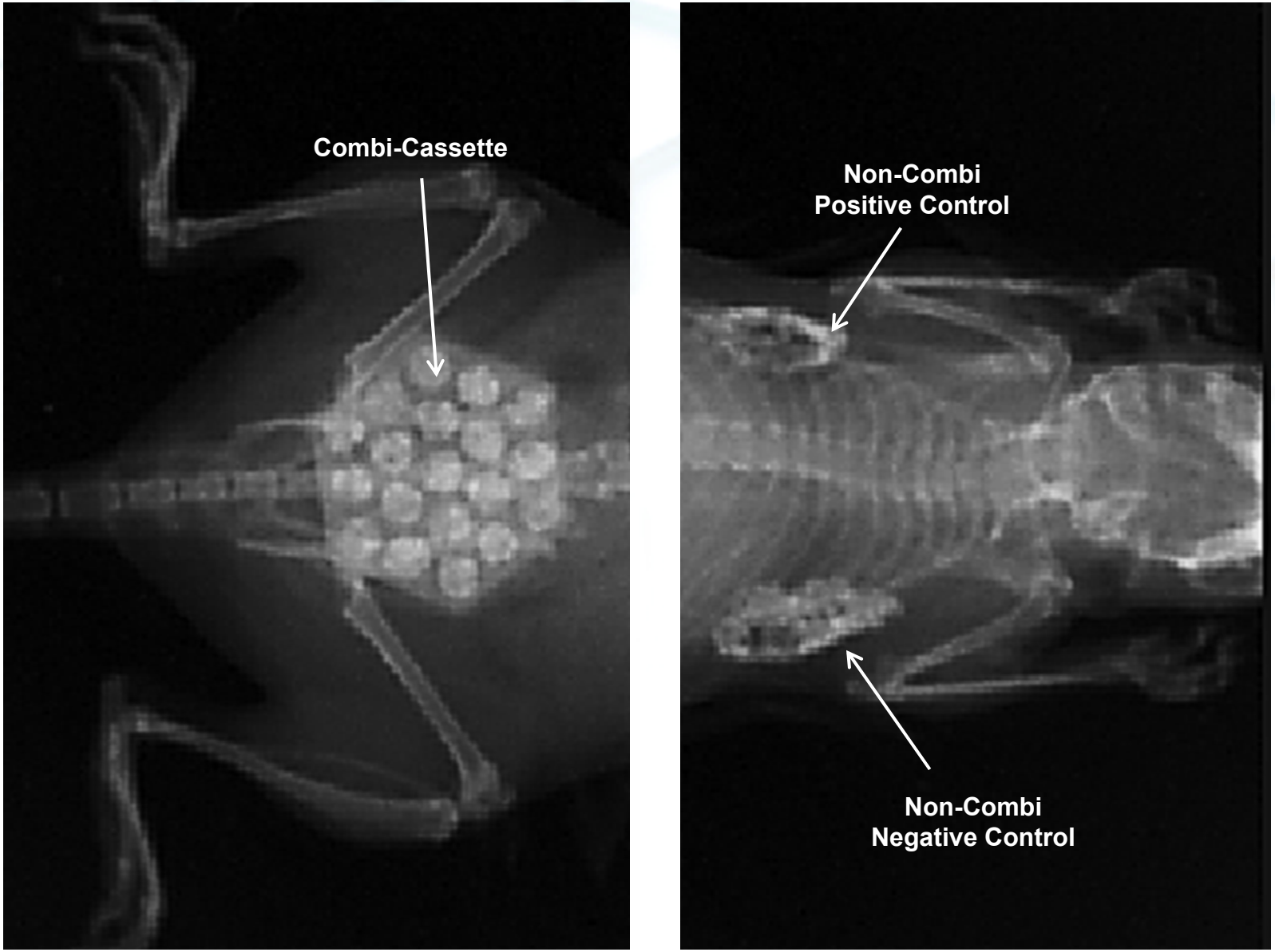


hBMSC Source:

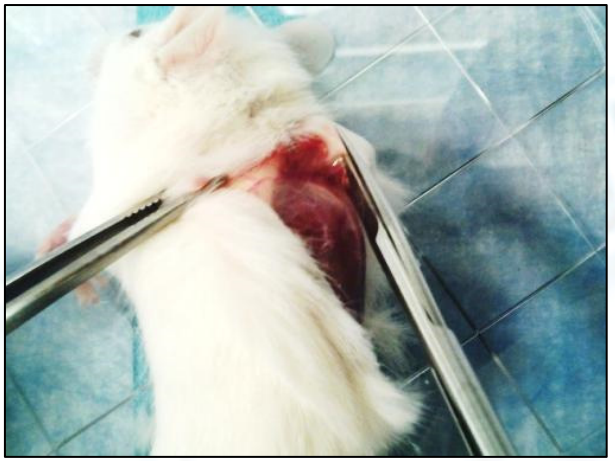
- Orthopedic surgical waste from a local clinic
- Spinal correction, scoliosis
- 11 yr female



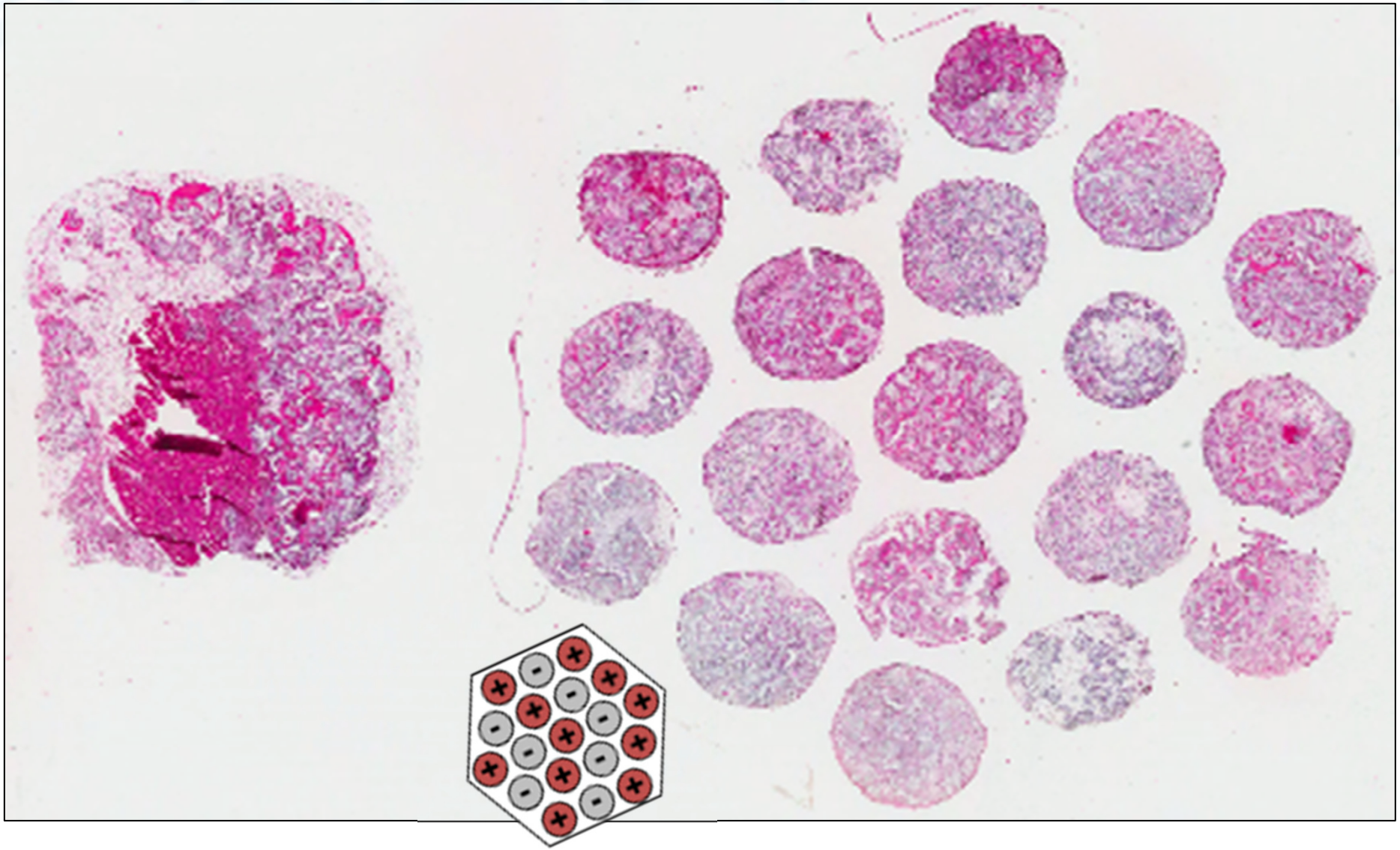
X-Ray Radiography after Surgery (hBMSCs)



Retrieval (hBMSCs)

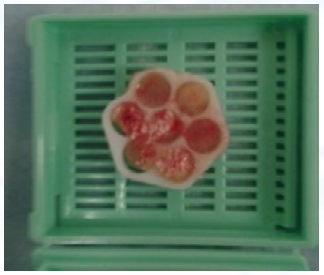


Histology (hBMSCs)



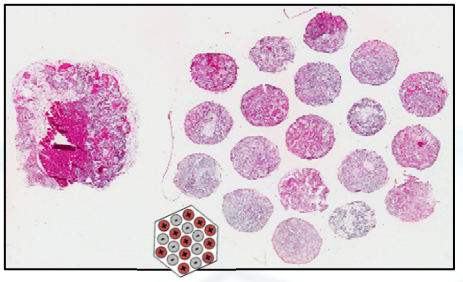
Histology More Efficient & Systematic with Combi (2 mos.)

Combi-Cassette



Non-Combi

Transplant Retrieval

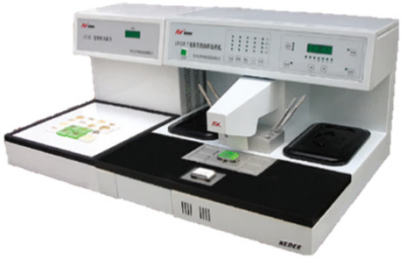


Tissue Fixation: 4%
Formaldehyde in PBS for 2 d

Tissue Demineralization:
0.25M EDTA for 10 d

Tissue Dehydration

Paraffin Embedding

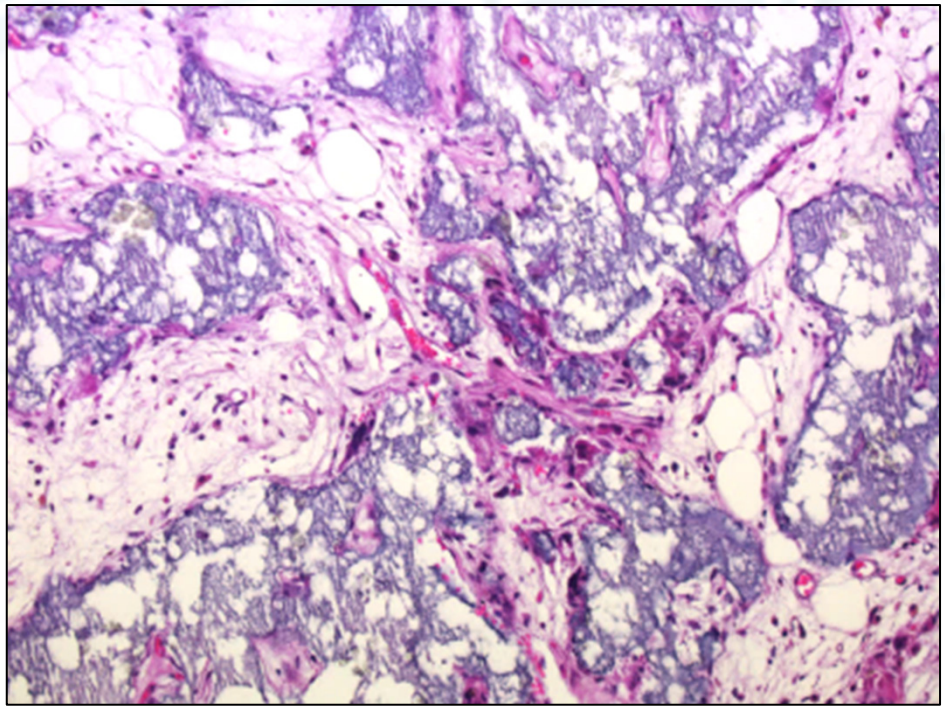


Tissue Sectioning

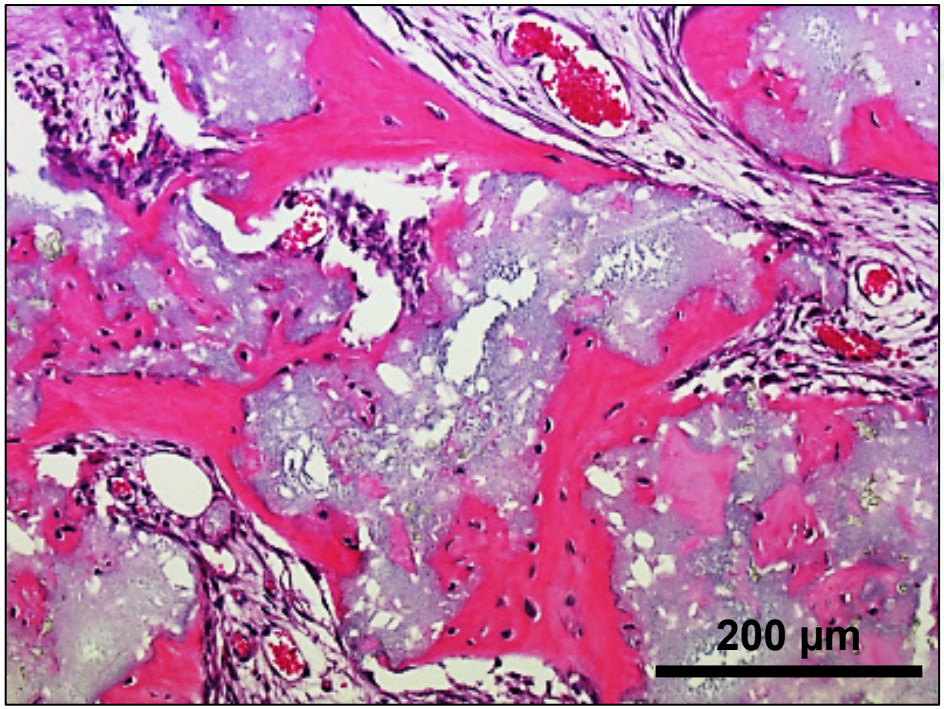
All implants can be fixed, demineralized, embedded, sectioned, mounted, stained & imaged together

Histology (hBMSCs)

Non-Osteogenic: HA/TCP + Fibrin



Osteogenic: hBMSCs + HA/TCP + Fibrin



Bone Scoring H&E Stained Slides, Semi-Quantitative Scale:

0 = no bone

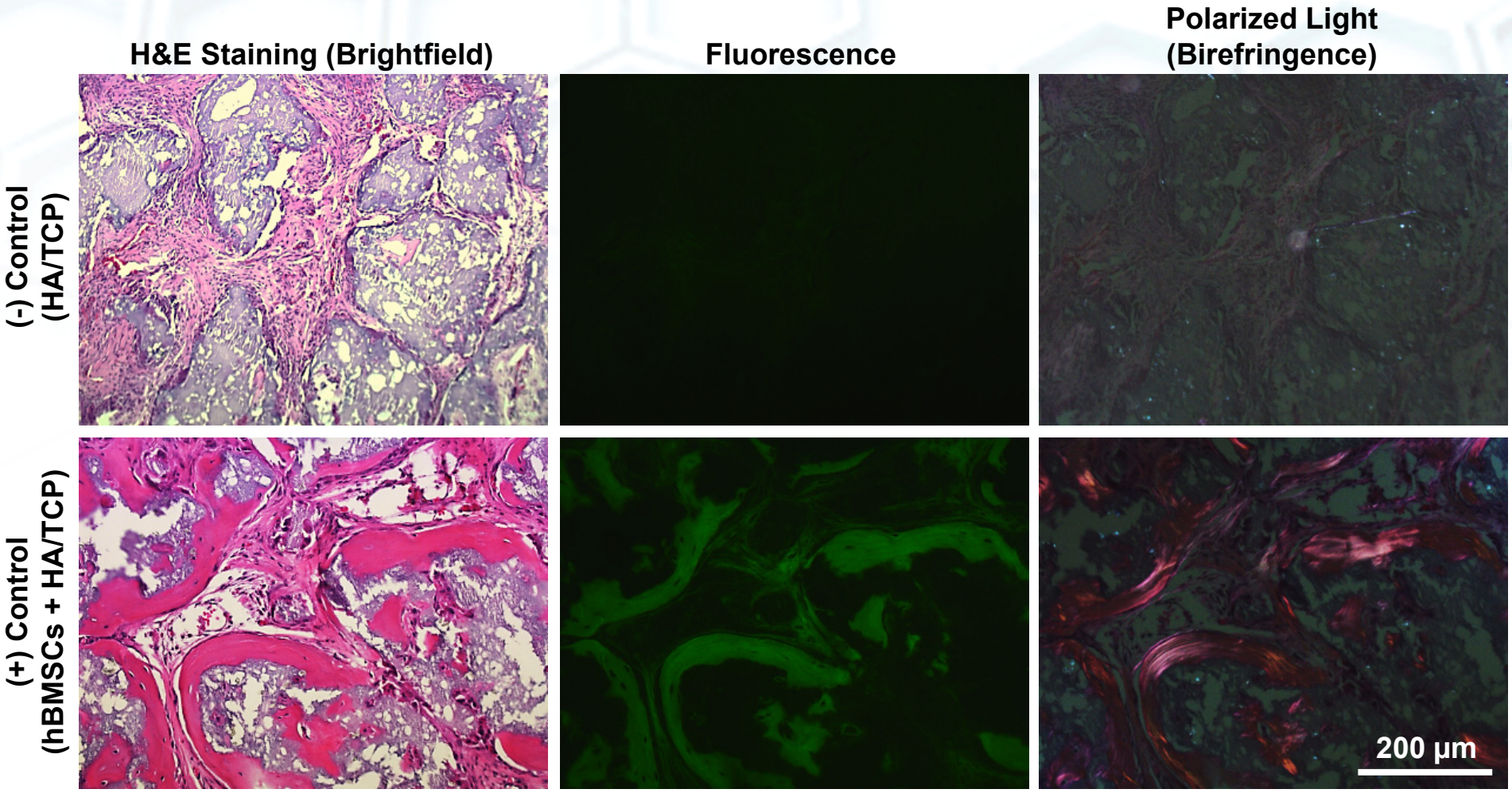
1 = minimal bone, just a single or a few bone trabeculae in one or a few sections

2 = low bone , multiple bone trabeculae in parts of some sections but only a small portion of the sections

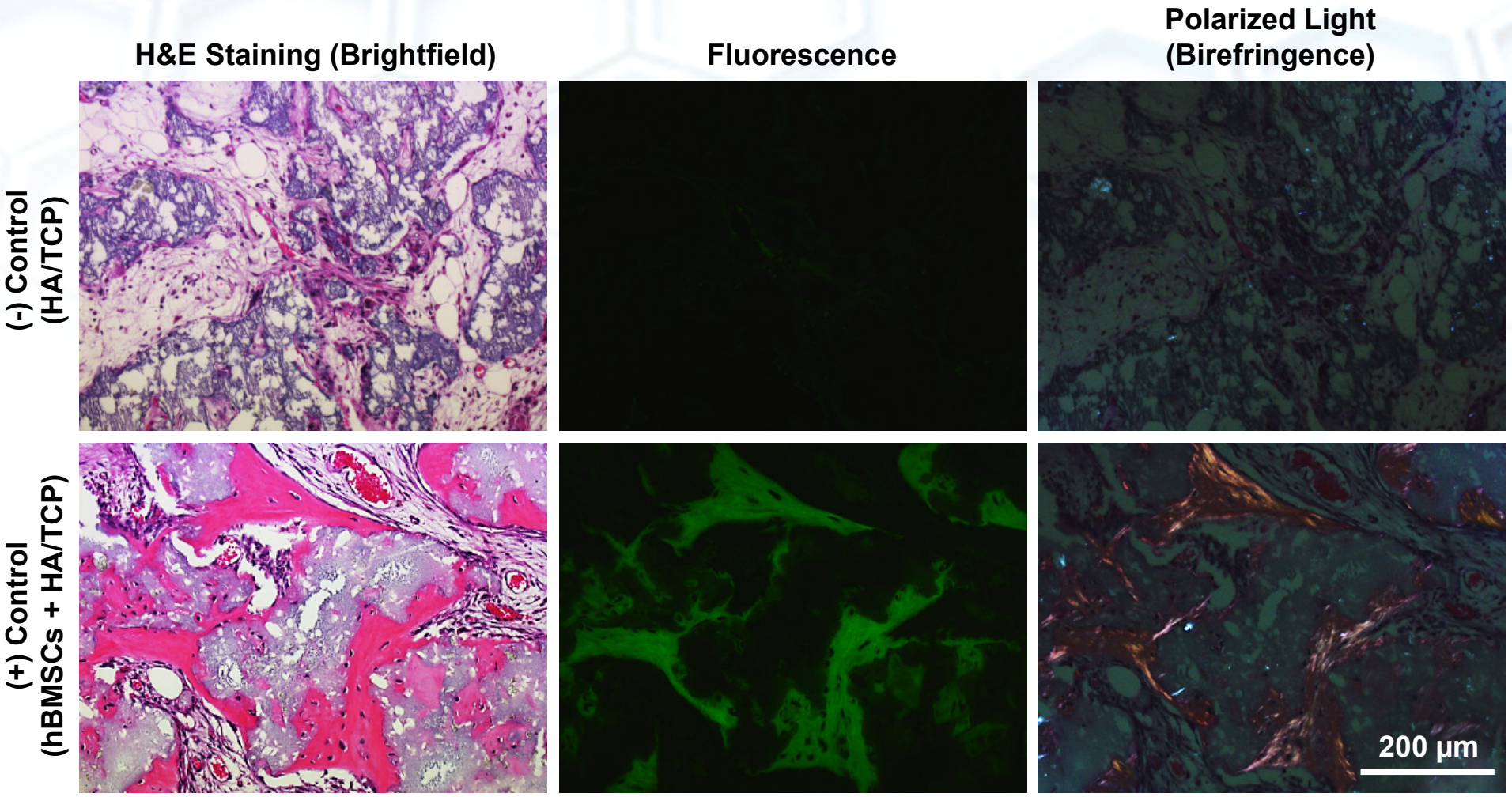
3 = moderate bone, bone occupies a significant portion but less than one half of most sections

4 = abundant bone, bone occupies greater than one half of each section

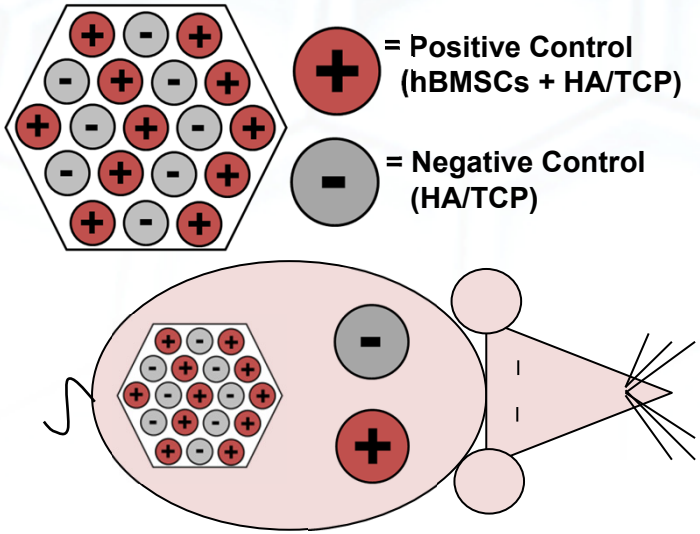
Histology for Non-Combi (hBMSCs)



Histology for 19-Well Combi-Cassette (hBMSCs)



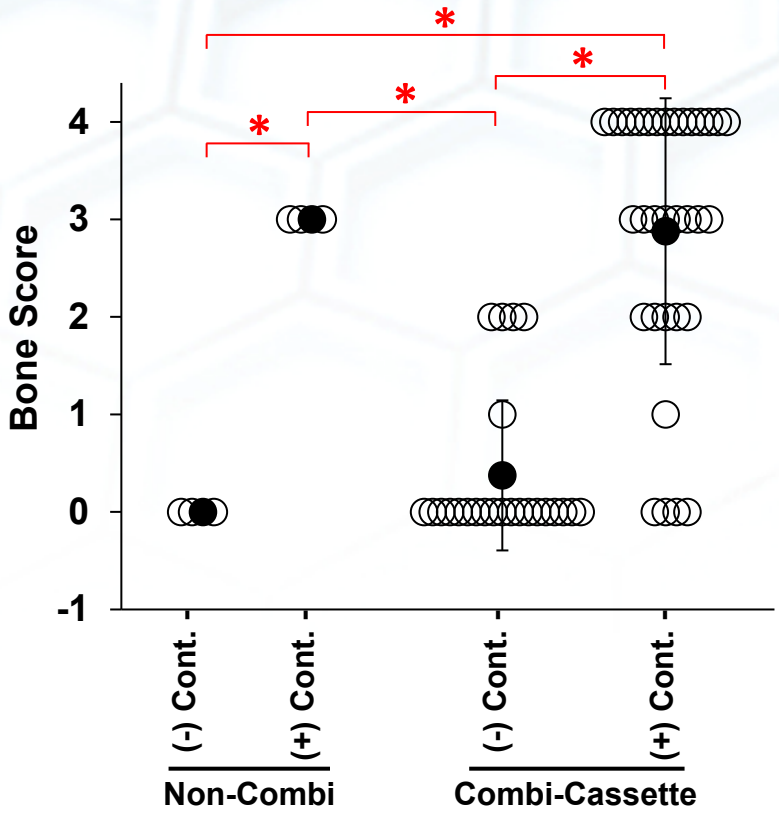
Bone Score Data (hBMSCs)



Well #	Description	Mouse 1	Mouse 2	Mouse 3
1	Combi Positive #1	1	4	4
2	Combi Positive #2	2	3	0
3	Combi Positive #3	3	3	2
4	Combi Positive #4	3	3	0
5	Combi Positive #5	4	4	2
6	Combi Positive #6	2	4	4
7	Combi Positive #7	4	4	3
8	Combi Positive #8	4	4	0
9	Combi Positive #9	4	3	0
10	Combi Positive #10	4	2	3
11	Combi Positive #11	4	4	4
12	Combi Negative #1	0	0	2
13	Combi Negative #2	0	0	0
14	Combi Negative #3	0	0	2
15	Combi Negative #4	0	0	0
16	Combi Negative #5	0	0	0
17	Combi Negative #6	0	0	0
18	Combi Negative #7	1	0	0
19	Combi Negative #8	2	2	0
--	Non-Combi Positive	3	3	3
--	Non-Combi Negative	0	0	0

Bone Scores (hBMSCs)

GOAL: Validate Combi-Cassette against Traditional Approach

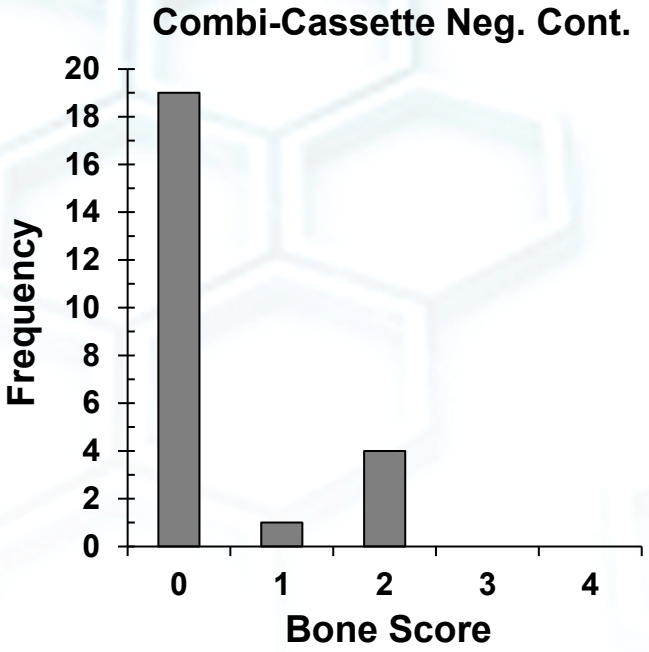


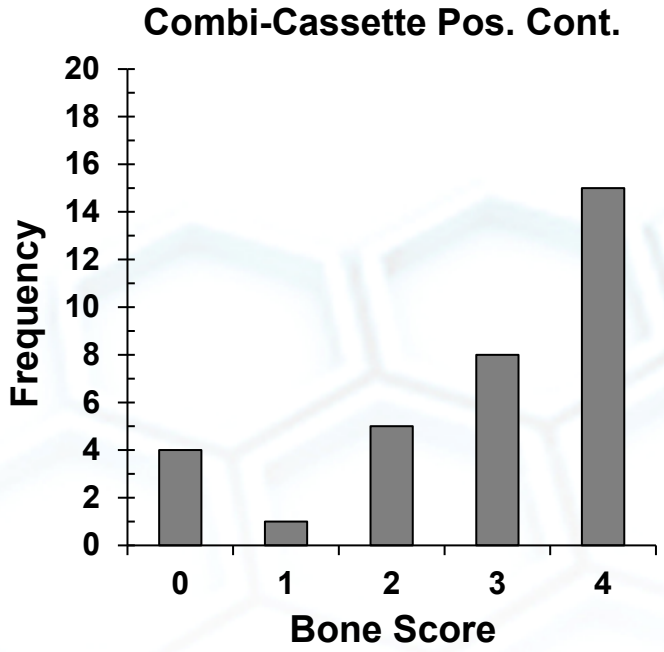
Open circles are individual data points, closed circles are means (with standard deviation).

Bone Scores (1-Way ANOVA with Tukey's)			
Comparisons			**P-Value**
Combi-Cassette Pos.	vs	Combi-Cassette Neg.	< 0.001
Combi-Cassette Pos.	vs	Non-Combi Pos.	0.998
Combi-Cassette Pos.	vs	Non-Combi Neg.	< 0.001
Combi-Cassette Neg.	vs	Non-Combi Pos.	0.002
Combi-Cassette Neg.	vs	Non-Combi Neg.	0.946
Non-Combi Pos.	vs	Non-Combi Neg.	0.009

Statistics	Interpretation
Positive controls were significantly different from negative controls (P < 0.009)	Evidence that the experiment worked correctly
No significant differences between combi and non-combi positive controls or between combi and non-combi negative controls (P > 0.95)	**Validates combi-cassette against traditional approach (non-combi)**

Histograms (hBMScs)





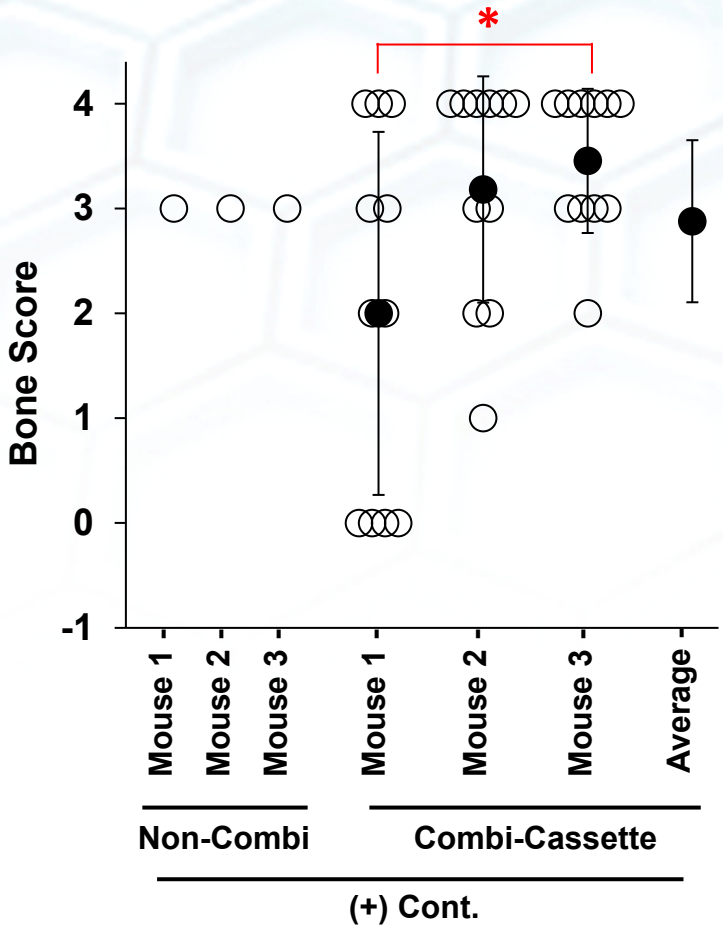
Histograms say that data is non-normal…do a nonparametric test?

(Kruskal Wallis uses medians which makes it less sensitive to shape of the distribution or differences in variance)

Bone Scores				
Comparisons			P-Value	
			1-way ANOVA w/Tukey's	Kruskal-Wallis
Combi-Cassette Pos.	vs	Combi-Cassette Neg.	< 0.001	< 0.001
Combi-Cassette Pos.	vs	Non-Combi Pos.	0.998	ns
Combi-Cassette Pos.	vs	Non-Combi Neg.	< 0.001	0.005
Combi-Cassette Neg.	vs	Non-Combi Pos.	0.002	0.016
Combi-Cassette Neg.	vs	Non-Combi Neg.	0.946	ns
Non-Combi Pos.	vs	Non-Combi Neg.	0.009	ns

Doesn't change the conclusions, but worth checking…

Mouse-to-Mouse Variability (hBMSCs)



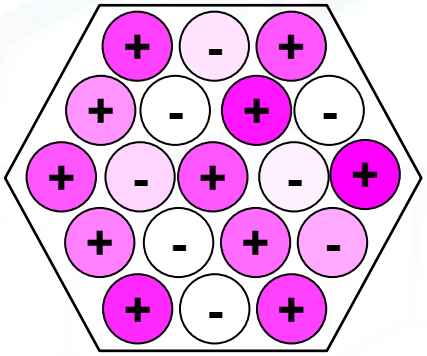
Open circles are individual data points, closed circles are means (with standard deviation).

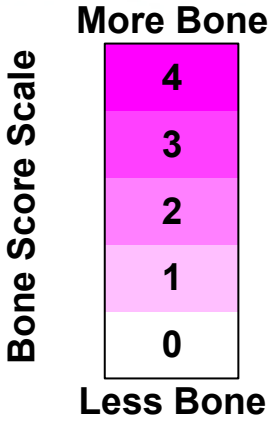
Bone Scores Mouse-to-Mouse Differences in Combi-Cassette Pos. Cont.				
Comparison			P-Value	
			1-way ANOVA w/Tukey's	Kruskal-Wallis
Mouse 1	vs	Mouse 2	0.87	> 0.05
Mouse 1	vs	Mouse 3	0.08	> 0.05
Mouse 2	vs	Mouse 3	0.03	0.04

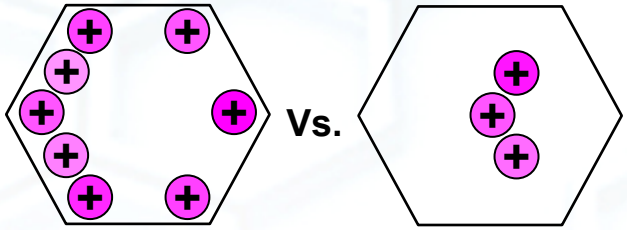
Statistics	Interpretation
Mouse 1 significantly different from Mouse 3 (P = 0.03)	Demonstrates that mouse-to-mouse variability can be detected

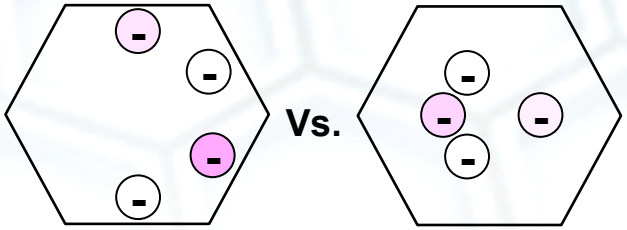
Does Well Number Affect the Results?

Heat map of bone scores by well (n = 3 mice)









Bone Scores				
Comparisons			P-Value	
			T-Test	Kruskal-Wallis
Outer Pos.	vs	Inner Pos.	0.98	0.64
Outer Neg.	vs	Inner Neg.	0.44	0.54

Statistics	Interpretation
No difference between outer & inner wells	Well position does not affect the results

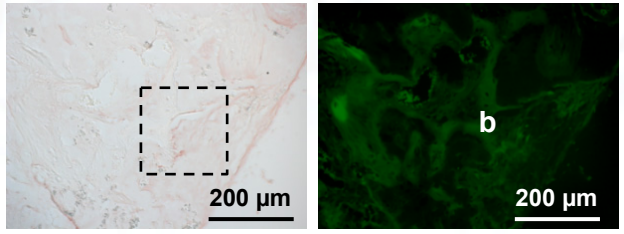
Human Cells Present in New Bone

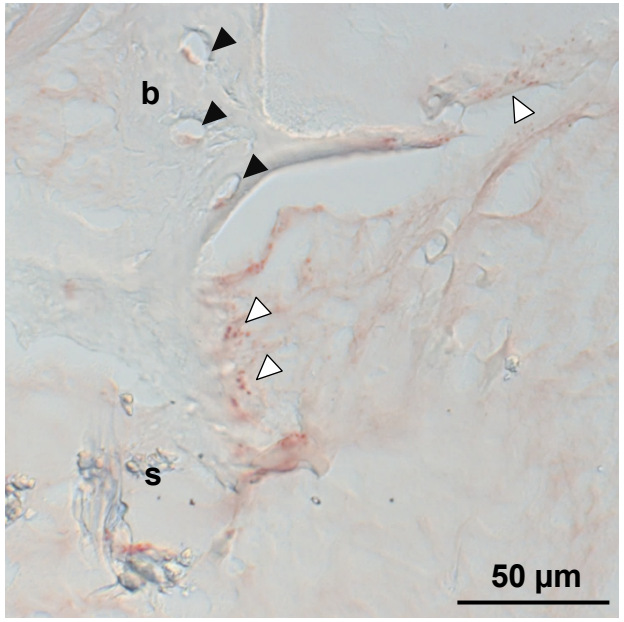
(MAB1273, anti-mitochondria antibody, surface of intact mitochondria, clone 113-1, EMD Millipore)

- Black Arrowheads = Osteocytes
- White Arrowheads = Osteoblasts
- b = Bone
- p = HA/TCP
- s = sinusoid
- Brown = Human Cells

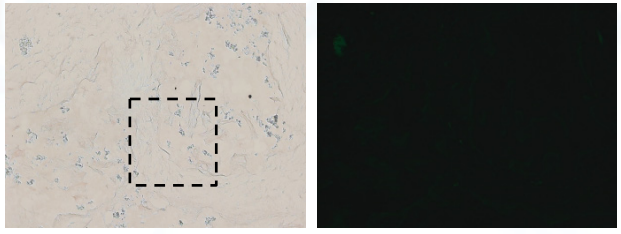
Combi-Cassettes

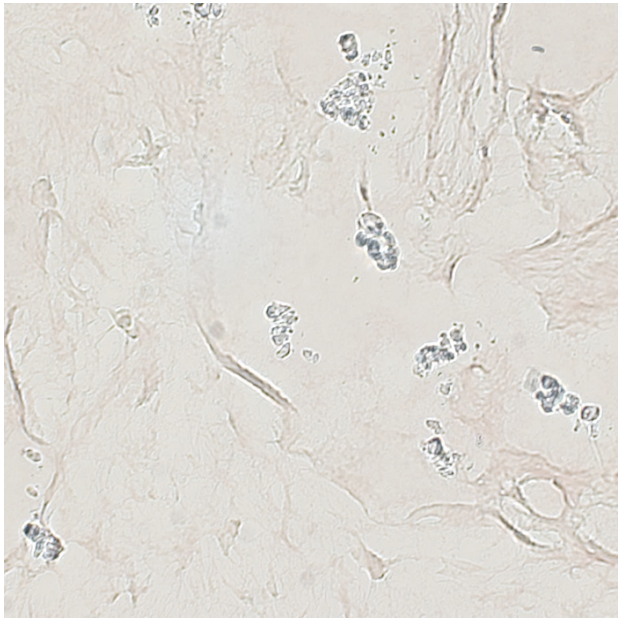
Pos. Cont. (hBMSC + HA/TCP)





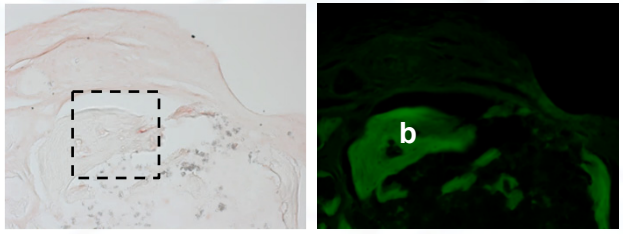
Neg. Cont. (HA/TCP)

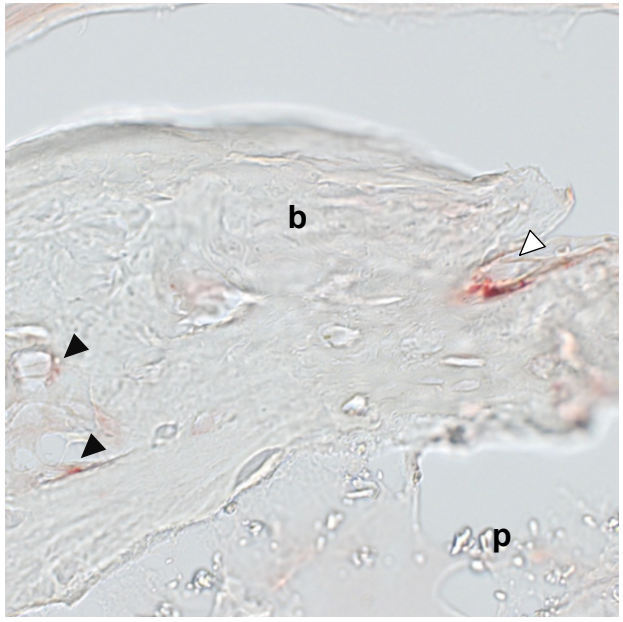




Non-combi

Pos. Cont. (hBMSCs + HA/TCP)

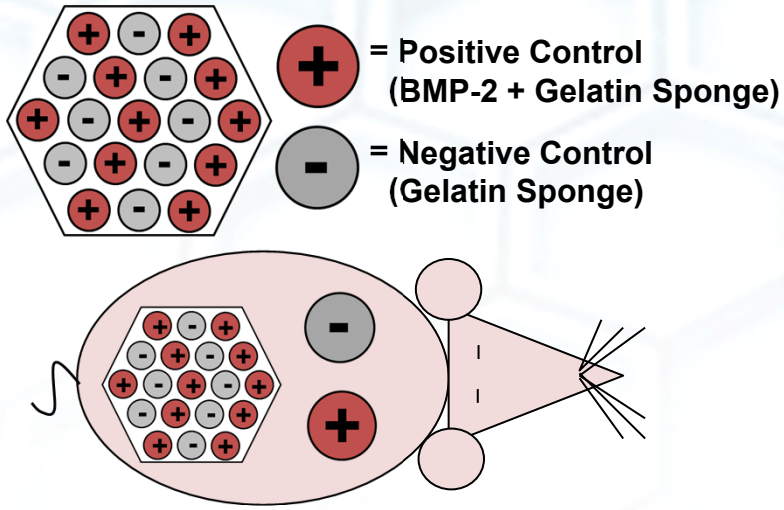


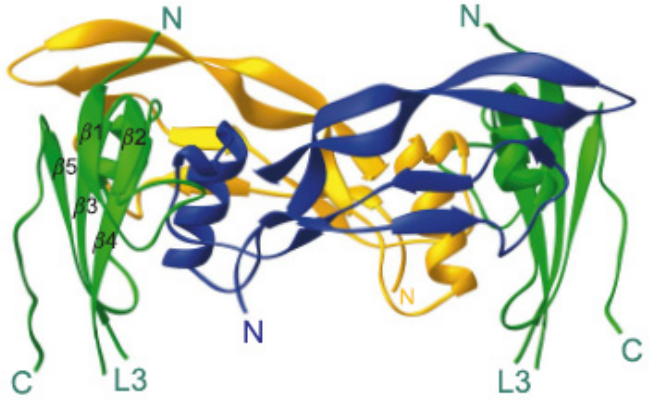


Growth Factor:

BMP-2

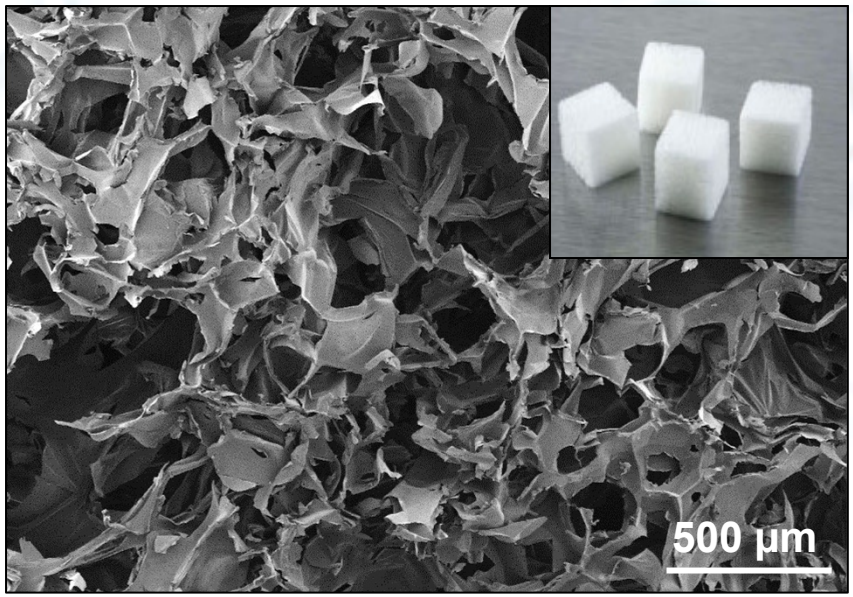
Growth Factor-Based Constructs: BMP-2 (8 wks)





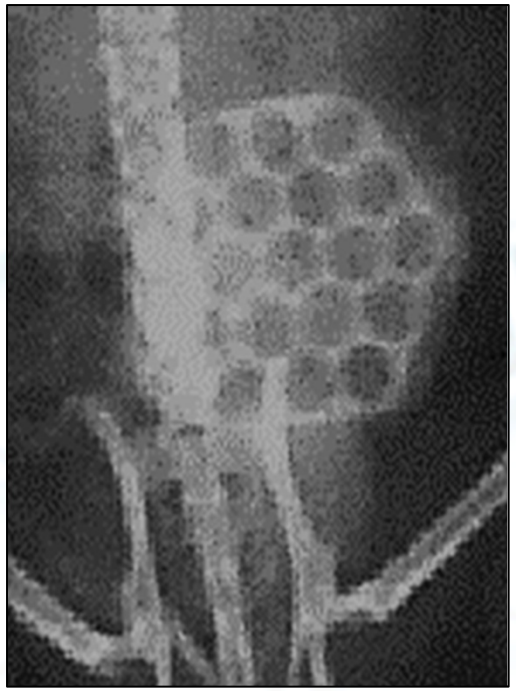
BMP-2 monomers (blue/gold) bound to BR1A (green)
[Kirsch et al., Nature Struct Biol 2000]

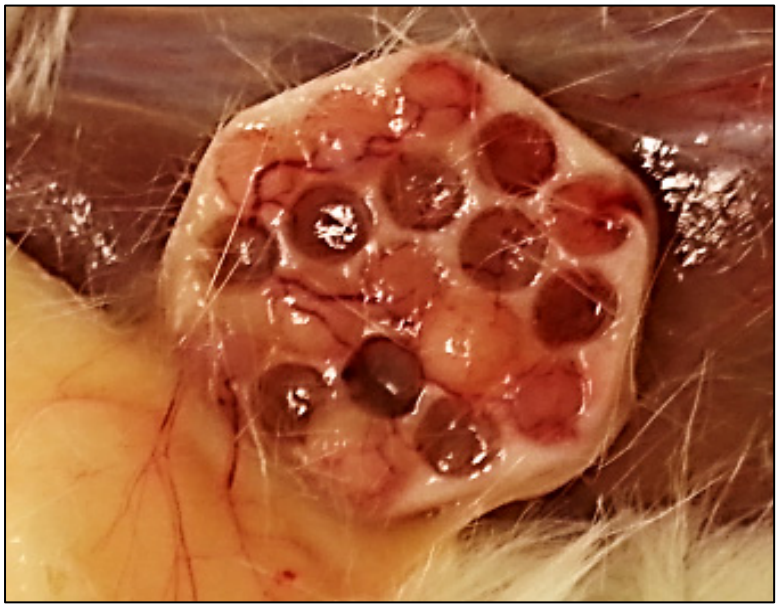
Gelatin Sponge (Gelfoam, Pfizer)

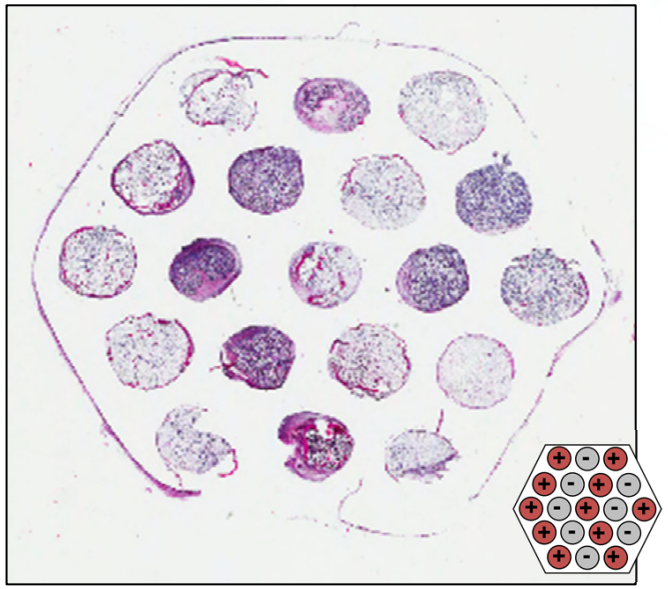


- BMP-2 = recombinant human bone morphogenetic protein-2 (eBioscience)
- 5 µg/scaffold
- Turns connective tissue cells into osteoprogenitor cells & can cause bone formation at non-bony sites

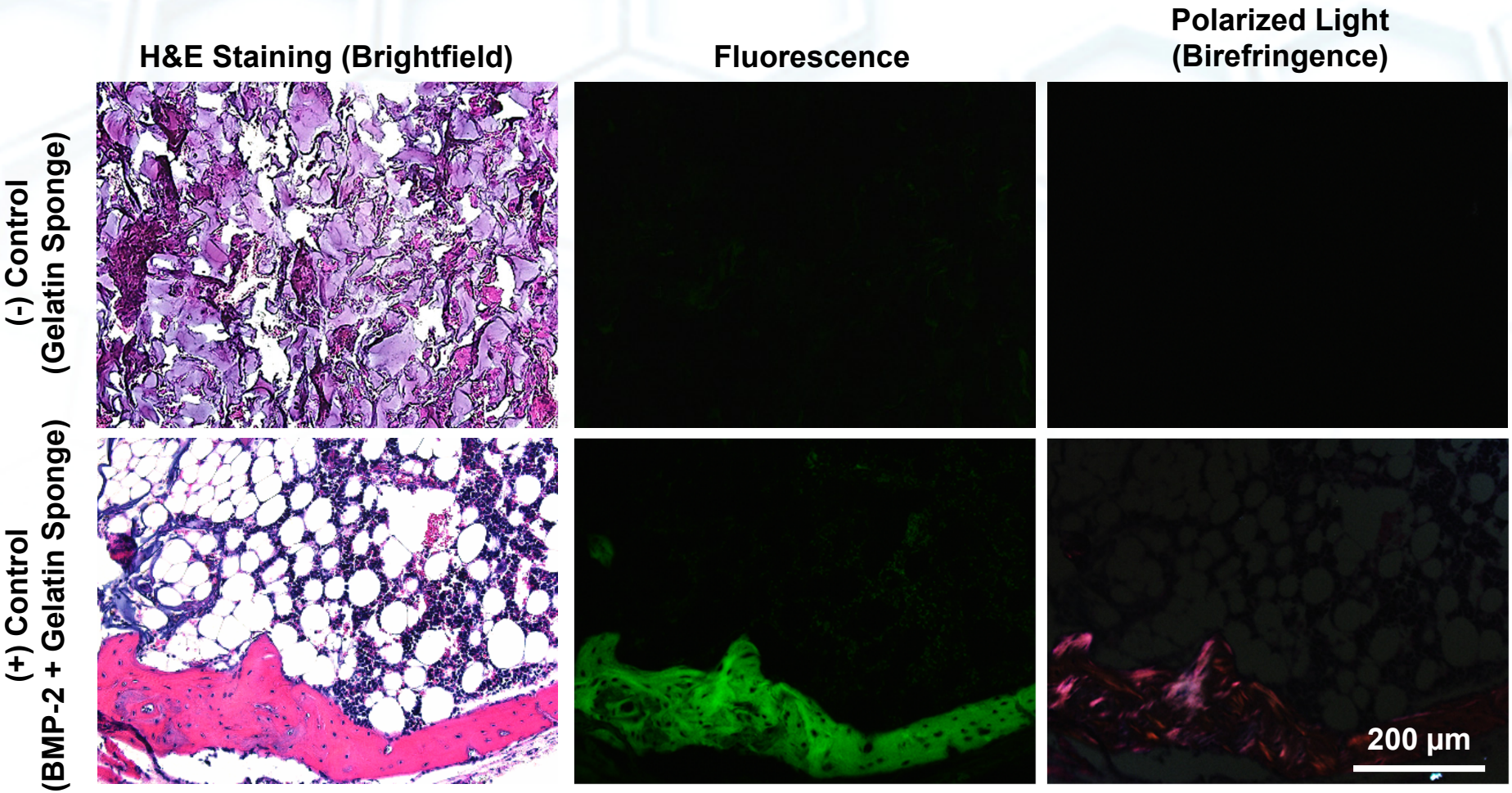
BMP-2



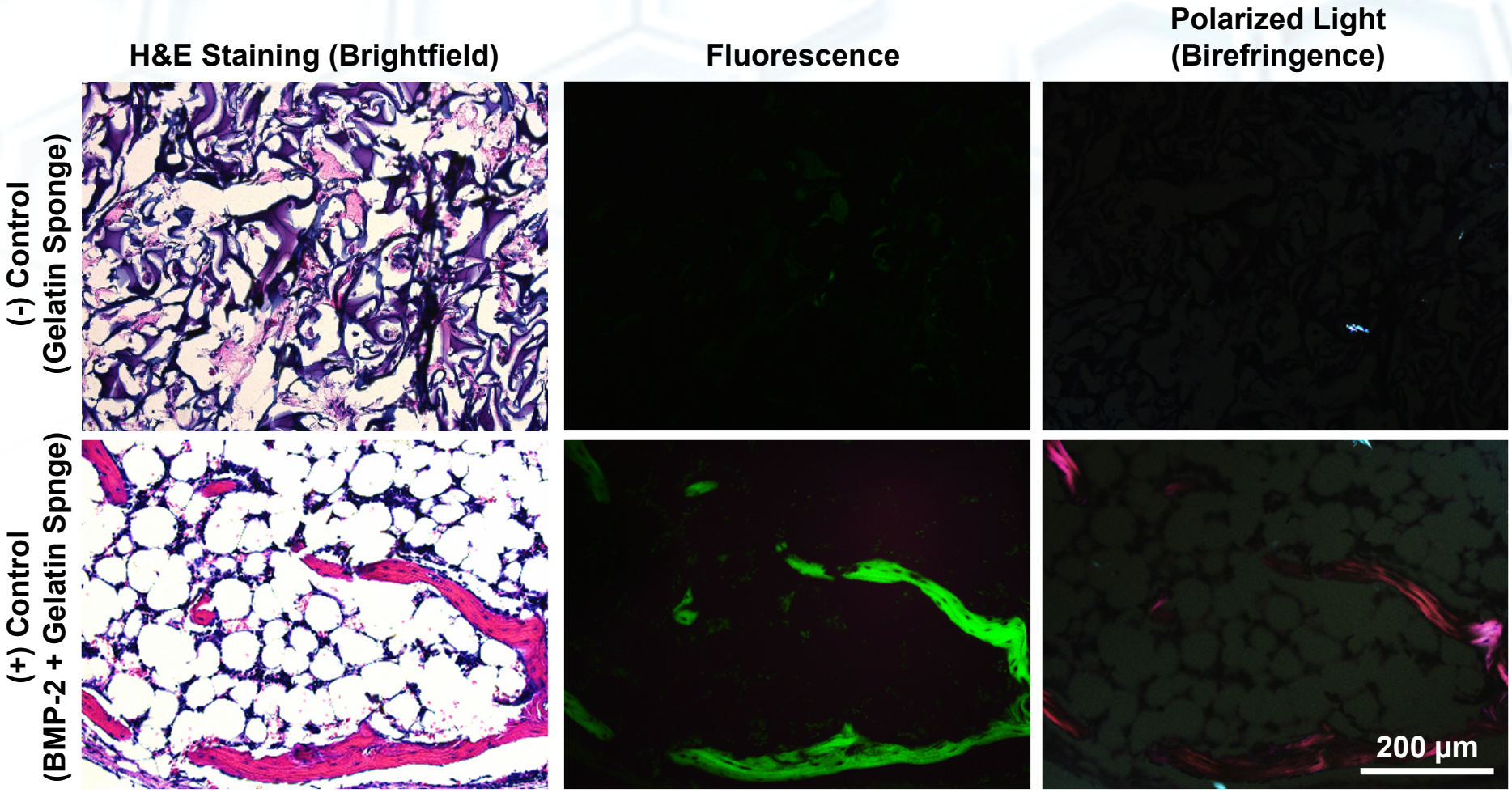




Histology: Non-Combi (BMP-2)

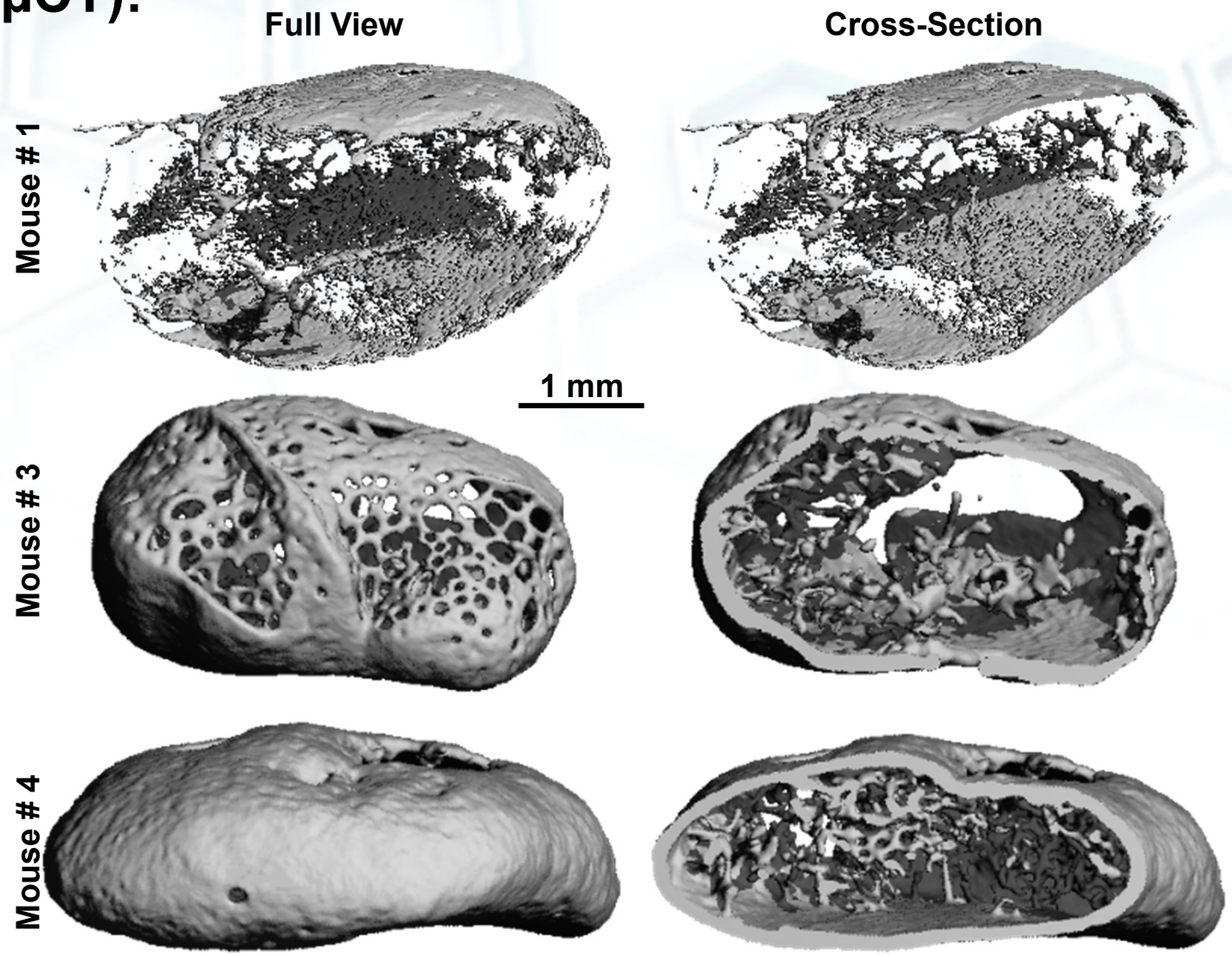


Histology: 19-Well Combi-Cassette (BMP-2)



X-Ray Computed Tomography (μCT): BMP-2

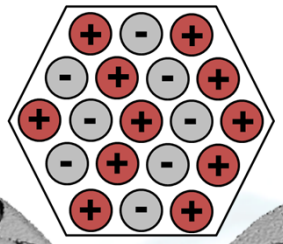
Non-Combi Positive Controls



Non-combi negative controls could not be assessed since they were fully resorbed.

µCT: BMP-2

19-Well Cassette Layout

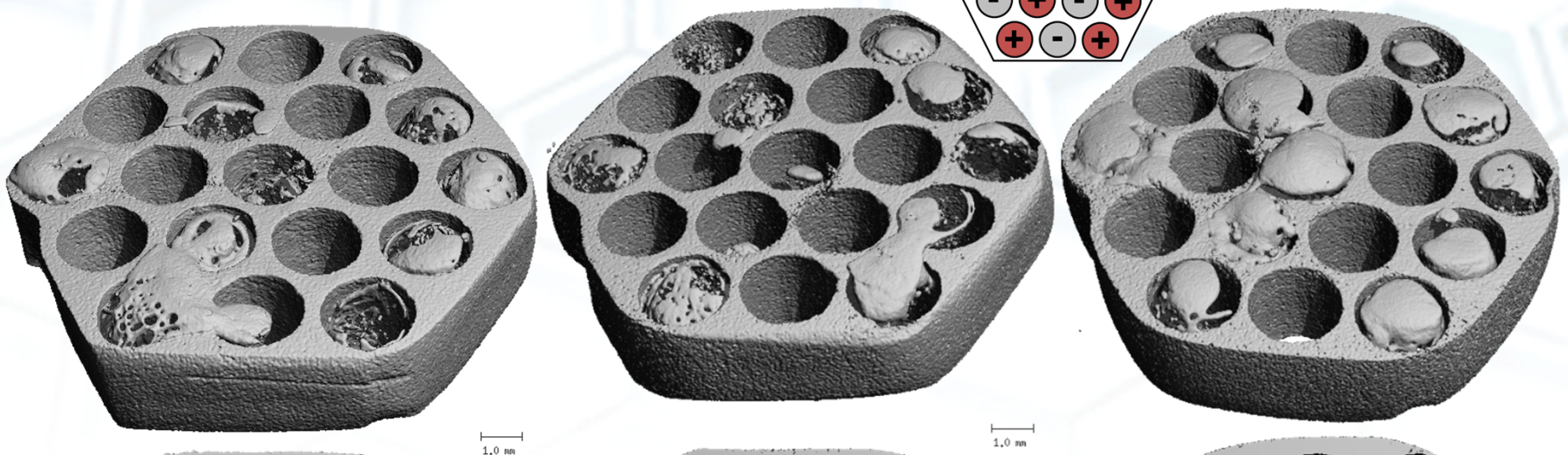


Mouse # 1

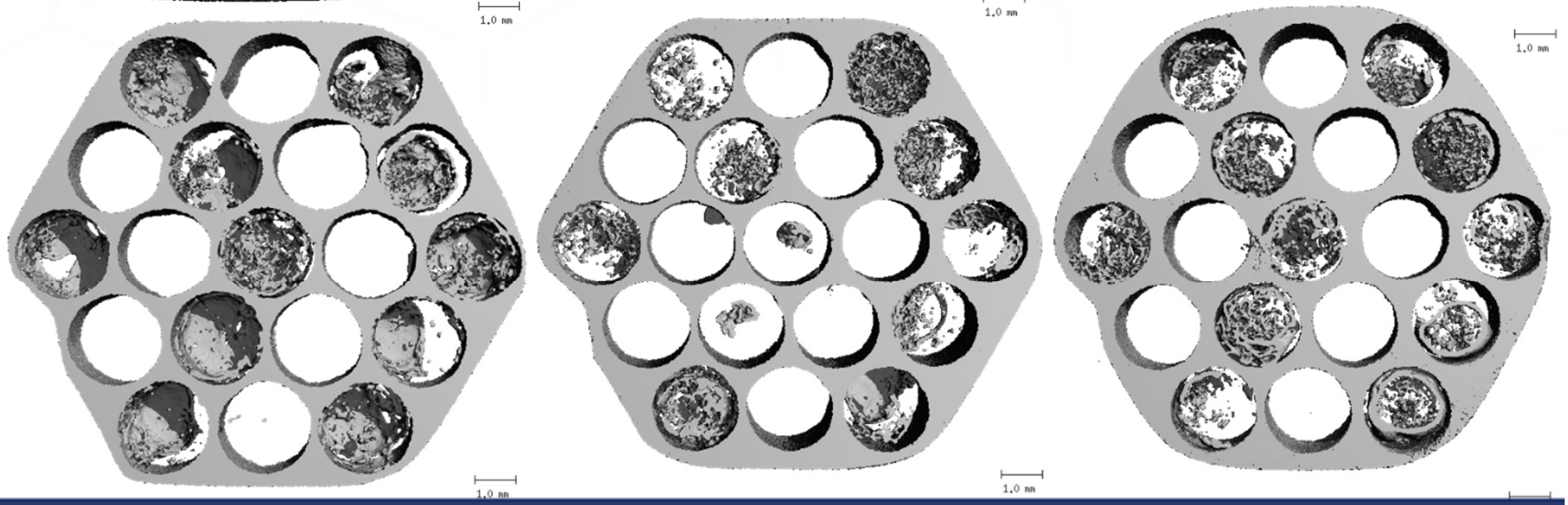
Mouse # 3

Mouse # 4

Full View



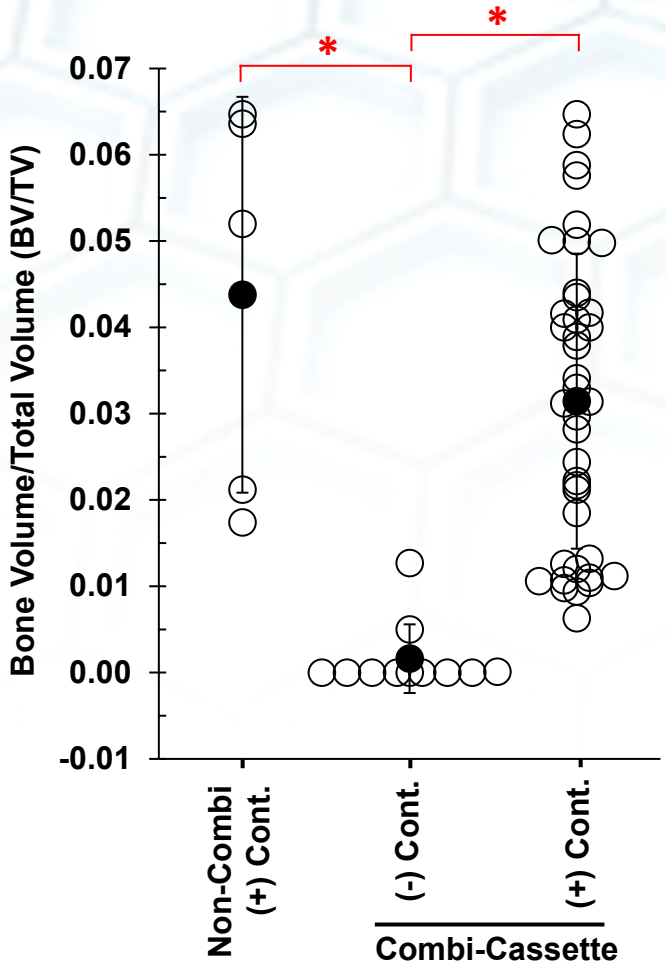
Cross-Section



µCT BV/TV (BMP-2)

Well #	Description	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6
1	Combi Positive	0.013	0.052	0.042	0.030	0.031	0.059
3	Combi Positive	0.013	0.024	0.058	0.042	0.006	0.040
6	Combi Positive						
8	Combi Positive						
9	Combi Positive	0.011	0.050	0.034	0.044	0.033	0.040
10	Combi Positive	0.009	0.044	0.010			
12	Combi Positive		0.038				
16	Combi Positive	0.021	0.022	0.010	0.028	0.011	0.062
17	Combi Positive	0.011		0.065	0.050	0.011	0.022
18	Combi Positive						
19	Combi Positive	0.031	0.012	0.019	0.041	0.039	0.050
2	Combi Negative					0.000	
4	Combi Negative					0.000	
5	Combi Negative				0.000		
7	Combi Negative	0.013		0.000	0.000		0.000
11	Combi Negative			0.005			
13	Combi Negative						0.000
14	Combi Negative						
15	Combi Negative			0.000			0.000
--	Non-Combi Positive	0.021	0.052	0.017	0.065	0.064	*Lost*
--	Non-Combi Negative	...unable to measure since they degrade…					

µCT (BMP-2)



Non-combi negative controls could not be assessed since they were fully resorbed.

BV/TV Values from µCT (1-Way ANOVA with Tukey's)			
Comparison			**P-Value**
Combi-Cassette Pos. Cont.	vs	Combi-Cassette Neg. Cont.	< 0.001
Combi-Cassette Pos. Cont.	vs	Non-Combi Pos. Cont.	0.151
Combi-Cassette Neg. Cont.	vs	Non-Combi Pos. Cont.	0.014

Statistics	Interpretation
Positive controls were significantly different from negative controls (P < 0.02)	Evidence that the experiment worked correctly
No significant differences between combi and non-combi positive controls (P > 0.95)	**MAIN GOAL: Validates combi-cassette against traditional approach (non-combi)**

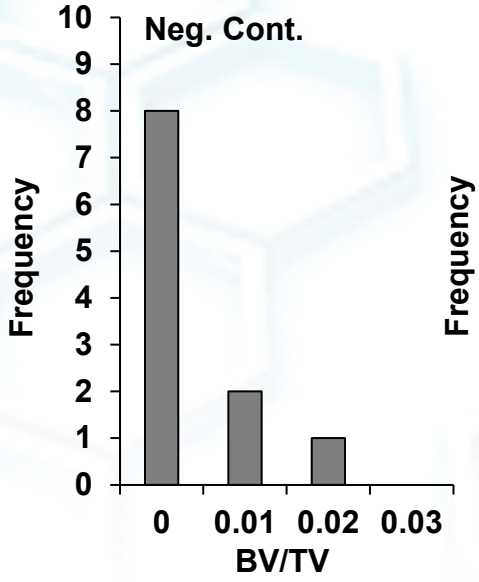
BV/TV Histograms (BMP-2)

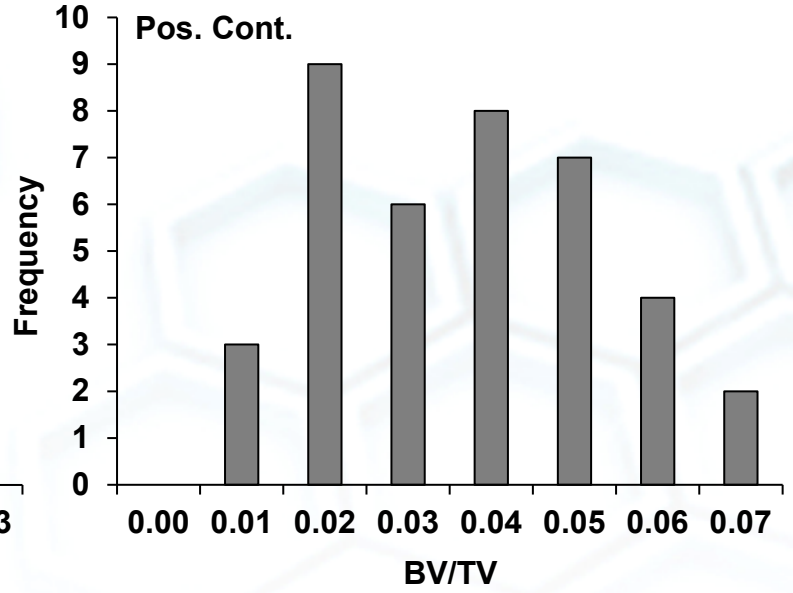
Normality Tests		
Test	**Neg. Cont**	**Pos. Cont.**
Anderson-Darling	P < 0.005	P = 0.892
Ryan-Joiner	P = 0.028	P > 0.100
Kolmogorov-Smirnov	P = 0.028	P > 0.15

Histograms say that neg. controls are non-normal so do a nonparametric test?

(Kruskal Wallis uses medians which makes it less sensitive to shape of the distribution or differences in variance)

Combi-Cassettes

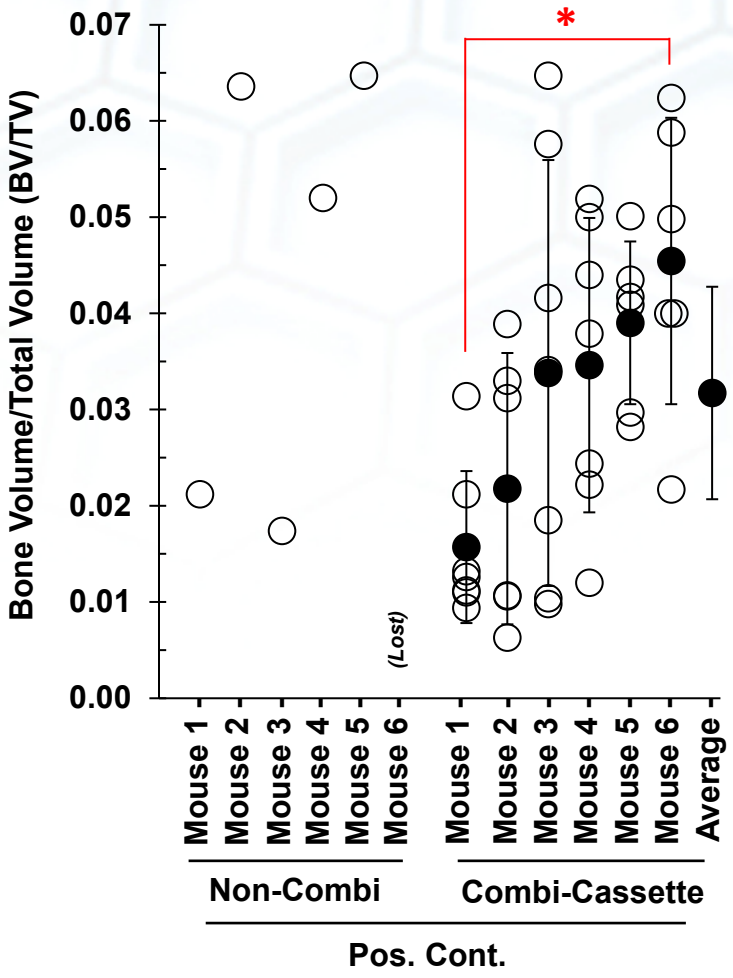




BV/TV				
Comparisons			**P-Value**	
			1-way ANOVA w/Tukey's	**Kruskal-Wallis**
Combi-Cassette Pos. Cont.	vs	Combi-Cassette Neg. Cont.	< 0.001	< 0.00001
Combi-Cassette Pos. Cont.	vs	Non-Combi Pos. Cont.	0.151	0.25
Combi-Cassette Neg. Cont.	vs	Non-Combi Pos. Cont.	0.014	< 0.0001

Doesn't change the conclusions, but worth checking…

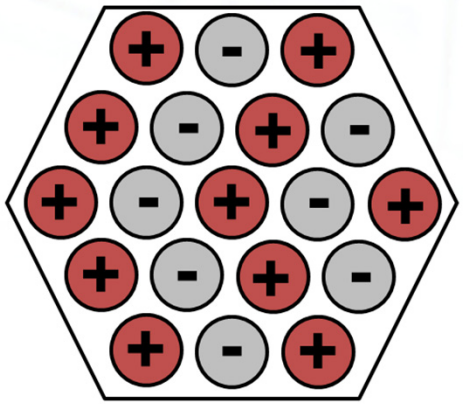
BMP-2 Mouse-to-Mouse Variability (BV/TV)

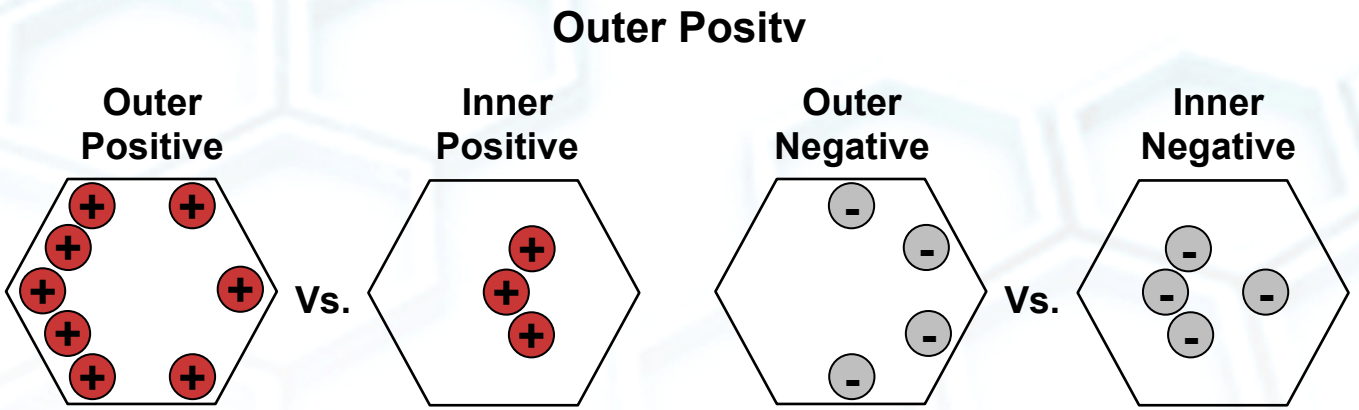


µCT (BV/TV): Combi-Cassette Pos. Cont.				
Comparison			P-Value	
			1-way ANOVA w/Tukey's	Kruskal-Wallis
Mouse 1	vs	Mouse 2	0.18	> 0.05
Mouse 1	vs	Mouse 3	0.21	> 0.05
Mouse 1	vs	Mouse 4	0.07	> 0.05
Mouse 1	vs	Mouse 5	0.97	> 0.05
Mouse 1	vs	Mouse 6	0.01	0.004
Mouse 2	vs	Mouse 3	1.00	> 0.05
Mouse 2	vs	Mouse 4	0.99	> 0.05
Mouse 2	vs	Mouse 5	0.64	> 0.05
Mouse 2	vs	Mouse 6	0.77	> 0.05
Mouse 3	vs	Mouse 4	0.99	> 0.05
Mouse 3	vs	Mouse 5	0.68	> 0.05
Mouse 3	vs	Mouse 6	0.73	> 0.05
Mouse 4	vs	Mouse 5	0.35	> 0.05
Mouse 5	vs	Mouse 6	0.98	> 0.05
Mouse 5	vs	Mouse 6	0.09	> 0.05

Statistics	Interpretation
Mouse 1 significantly different from Mouse 6 (P = 0.01)	Demonstrates that mouse-to-mouse variability can be detected

Does Well Number Affect the Results (BMP-2)?



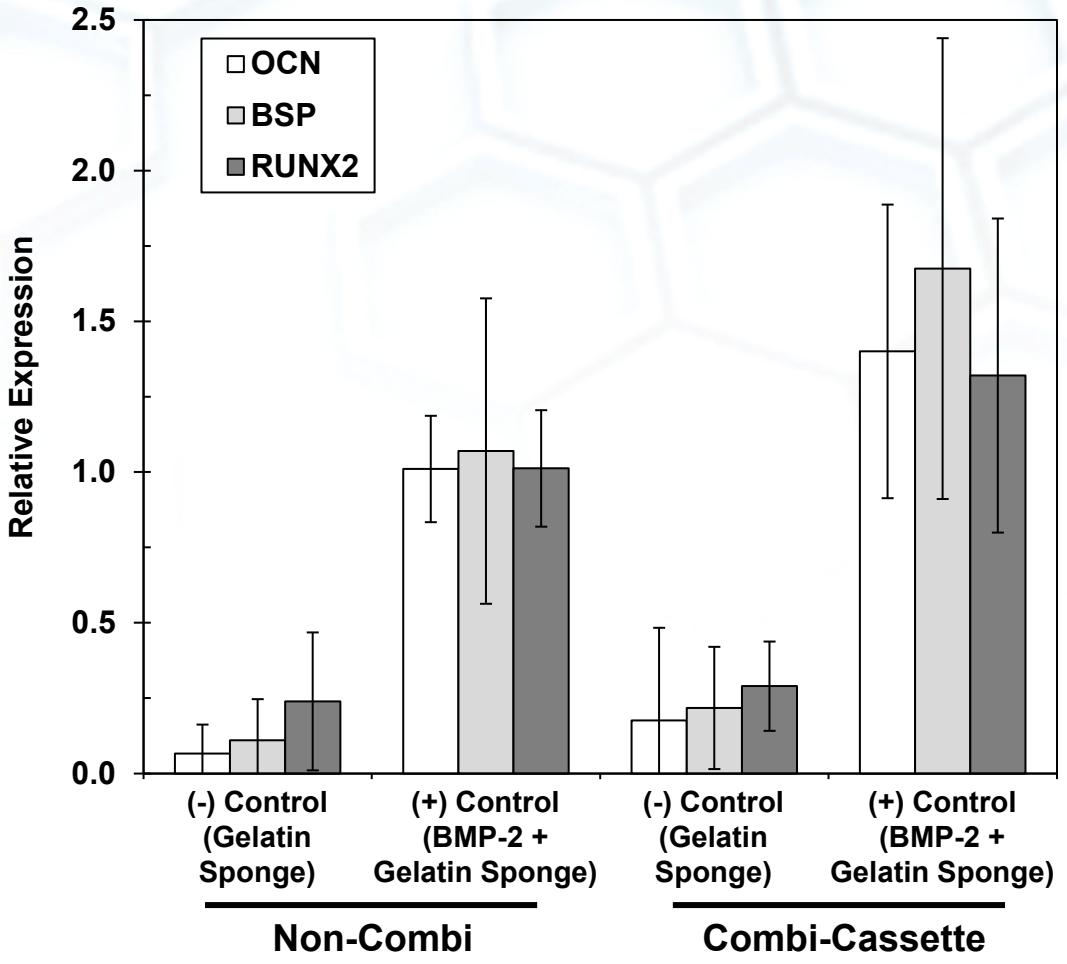


Bone Scores				
Comparisons			P-Value	
			T-Test	Kruskal-Wallis
Outer Pos.	vs	Inner Pos.	0.86	0.88
Outer Neg.	vs	Inner Neg.	0.84	0.34

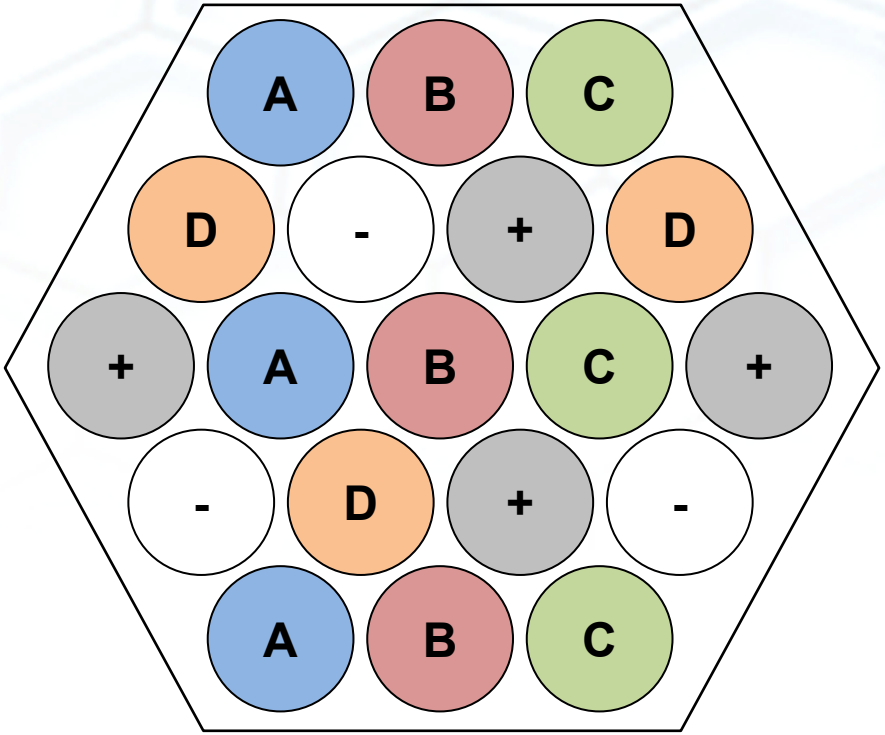
Statistics	Interpretation
No difference between outer & inner wells	Well position does not affect the results

PCR for Osteogenic Markers

- Data are from 3 mice
  - n = 3 for non-combi samples
  - n was between 4 and 8 for combi-cassette
- For all three genes, there were no significant differences between positive controls for combi-cassette versus non-combi (1-way ANOVA with Tukey's test, $P > 0.44$)
- For combi-cassette, positive control was significantly different from negative control for all three genes (1-way ANOVA with Tukey's test, $P < 0.005$)



Recommended Design: Six Treatments in Triplicate

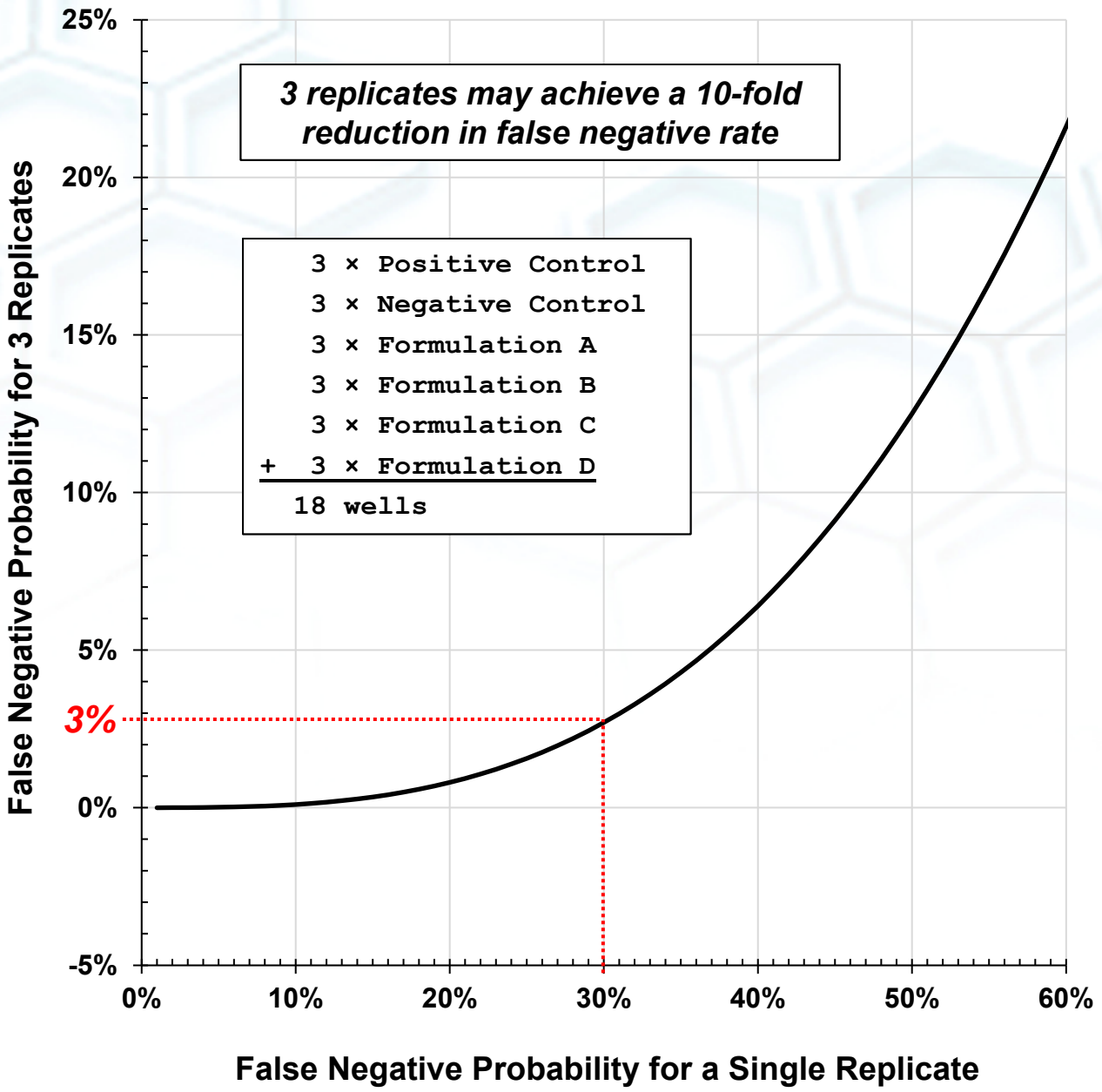


	3 × Positive Control
	3 × Negative Control
	3 × Formulation A
	3 × Formulation B
	3 × Formulation C
+	3 × Formulation D
	18 wells

False Negative Rate

- Estimated false negative rate is 30% for a single replicate (30% chance of missing an osteogenic formulation)
- If using combi-cassettes for triplicates, then false negative drops to 3% ≈ 30% × 30% × 30%

We ignore false positive rate since it is unlikely.

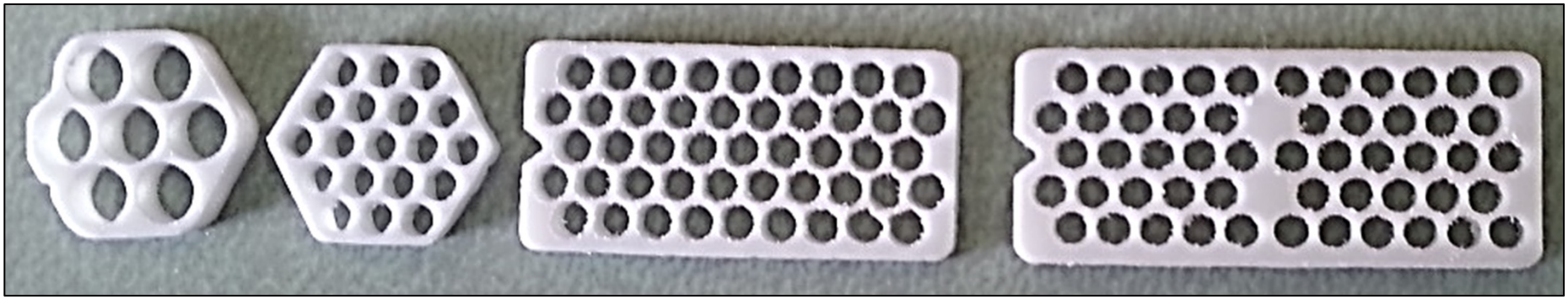


Combi-Cassette vs. Non-Combi

Combi-Cassette	Non-Combi
19 formulations per mouse	4 formulations per mouse
Replicates enable statistics: i) mouse-to-mouse variability & ii) differences between formulations in the same mouse	Single replicate prevents statistical analysis
When an implant is fully resorbed, the region inside the wells can be analyzed to provide background data	When an implant fully resorbs, data collection is prohibited since only skin & muscle remain
Histology more systematic: entire cassette can be fixed, demineralized, embedded, sectioned, mounted, stained, imaged & scored	Histology less consistent: each implant must be individually fixed, demineralized, embedded, sectioned, mounted, stained & imaged
Volume of interest is systematically defined by wells	Volume of interest ill-defined
Holds implants in place	Implants can move around under the skin after implantation
Uses a smaller dose (advantageous when materials are limited)	Larger dose (disadvantageous when materials is limited)
Smaller dose may reduce assay sensitivity	Larger dose may increase assay sensitivity
Neighboring wells can influence one another	Implants separated by longer distance
Shields sides of implants from the microenvironment	All sides of implants exposed to the microenvironment
Materials may fall out of cassette	Samples cannot fall out

**Green shading indicates an advantage*

Other Designs



Results Summary

hBMSCs

- Histology
- Bone scoring
- Human mitochondrial staining

BMP-2

- Histology
- µCT (BV/TV)
- PCR

Conclusions

- 19-wells (4.75-fold increase over non-combi 4 implants/mouse)
  - Demonstrated for cell-based (hBMSCs) & growth-factor based (BMP-2)
  - Could detect animal to animal variability
  - Well position was not a factor
  - Histology more systematic
  - Advantages of having wells:
    - Makes PCR & µCT more systematic since tissue volumes are more consistent
    - Defines volume for analysis for when implants fully resorb (for statistics)
- Recommended design: 6 treatments X 3 replicates = 18 wells
  - Each mouse gets 3 pos. cont., 3 neg. cont. & 4 experimental formulations
  - Different formulations can be statistically compared in the same mouse
  - False negative rate drops from 30% (single replicate) to 3% (triplicate)

Thank you!