

Whole-Mount Immunofluorescence And Fluorescence In Situ Hybridization For Protein And mRNA Labeling In Skeletally Mature Mouse Bones

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INTRODUCTION: Osteocytes sense external forces and initiate the bone mechanoadaptation response by regulating their molecular expression. RNA sequencing, 2D immunolabeling, and 2D in situ hybridization have been used to investigate changes in osteocyte genes and proteins expression in response to loading. However, these approaches do not preserve the 3D spatial information. Under loading, we know that mechanical environments vary along the bone length and result in location-dependent biological responses. There is a need for 3D tools to investigate molecular expression in osteocytes in their spatially preserved location. Tissue clearing protocols enable the optical clearing of various organs including bones. This approach allows visualization of bone blood vessels, nerves, and endogenous fluorescence. However, labeling of the osteocyte mRNA and proteins in whole-mount bone remains challenging due to the osteocytes' location within the dense bone matrix. In this work, we present a novel approach for 3D immunofluorescence and mRNA staining in whole mount mouse bone.

METHODS: All experiments were approved by IACUC. Tibiae from skeletally mature C57BL/6 mice were collected, fixed with paraformaldehyde, and decalcified using EDTA. *mRNA labeling:* For Fluorescent In Situ Hybridization (FISH), DNA probes were designed to recognize DMP1 mRNA transcripts, a gene involved in matrix mineralization, and SOST, a gene which is downregulated during bone formation. Both genes are expressed by osteocytes. The signal was amplified via fluorescent DNA hairpins recognizing SOST ($\lambda_{Ex}/\lambda_{Em}$: 650/671 nm) and DMP1 ($\lambda_{Ex}/\lambda_{Em}$: 590/617 nm) probe sets. *Protein labeling:* For immunofluorescence, samples were preserved using SHIELD (LifeCanvas Technologies) that protects tissue structure and molecular components. Then, an enzymatic matrix permeabilization step was performed to enable antibody penetration. Goat antibodies, immunoglobulin G (IgG), against sclerostin, were incubated with the samples for 4 days. The signal was amplified using secondary antibodies for 4 additional days. As an alternative approach, we also used customized fluorescent nanobodies to target and label sclerostin in bone ($\lambda_{Ex}/\lambda_{Em}$: 650/671 nm). This approach does not require matrix permeabilization and incubation times are reduced. Nuclear staining was achieved using Oxazole Yellow Homodimer ($\lambda_{Ex}/\lambda_{Em}$: 491/508 nm). All samples were mounted in agarose gel and their refractive index were matched to 1.52. Images were acquired using lightsheet microscopy.

RESULTS: Fluorescent signals from targeted mRNA transcripts in osteocytes were imaged throughout the bone (Figure 1). We also successfully labeled sclerostin protein in whole-mount mouse bone using traditional antibodies and nanobodies. Traditional IgG targeting sclerostin showed full penetration of the antibodies through the cortical thickness of a mouse tibia midshaft only when matrix was permeabilized (Figure 3). Full penetration was also observed using nanobodies targeting sclerostin in whole-mount mouse femur (Figure 2).

DISCUSSION: Matrix permeabilization has been found to be critical for optimal penetration of the IgG. In addition, the size of the sample, permeabilization solution concentration, incubation time, and presence of the bone marrow influenced the final penetration. Alternatively, nanobodies represent a faster but more costly alternative.

SIGNIFICANCE: To our knowledge, it is the first whole-mount immunolabeling protocol for skeletally mature mouse bones. This method will allow 3D investigation of bone cells molecular signal and enable correlation with their 3D mechanical environment. Our approach will provide new insight into bone mechanoadaptation and mechanotransduction. Future work will compare protein and mRNA expression in loaded versus control mouse tibiae and establish methods to quantify expression.

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