Optimization of Live-Cell Calcium Imaging to Visualize DREADD-induced Morphological Changes in Chondrocytes

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Introduction: Calcium represents a key secondary messenger regulating chondrocyte/cartilage metabolism. Although not classically considered excitable cells, chondrocytes leverage chemically- and mechanically-sensitive calcium (Ca^{2+}) signaling cascades within the context of cartilage development, homeostasis, and degradation. Our lab previously demonstrated that hM3Dq, a chemogenetic Designer Receptor Exclusively Activated by Designer Drugs (DREADD), can be utilized to spatiotemporally induce Ca^{2+}fluxes and oscillations within chondrocyte-like ATDC5 cells. Specifically, hM3Dq is a modified Gαq receptor that can activate the endogenous PLCβ–IP3–ER signaling pathway upon binding with its synthetic ligand clozapine-N-oxide (CNO; or similar derivatives). hM3Dq activation by CNO induces unique oscillatory Ca^{2+} signaling activation in ATDC5 cells not previously observed with the activation of Ca^{2+} channels, such as TRPV4. We've shown that such oscillatory Ca^{2+} signaling behavior upregulates anabolic gene expression and promotes cartilage neo-tissue formation, even in the absence of differentiation factors. However, the specific intracellular signaling mechanisms linking this oscillatory Ca^{2+} activity to anabolic gene expression remain poorly understood. In this study, we: 1) establish an improved live-cell imaging protocol to better capture oscillatory Ca^{2+} signaling behavior by mitigating photobleaching, 2) evaluate the use of a safer and more potent synthetic ligand, deschloroclozapine (DCZ), for its ability to induce oscillatory Ca^{2+} cascades, and 3) investigate if hM3Dq and Ca^{2+} signaling drive ATDC5 cell contractility following DREADD activation.

Methods: Live cell calcium signaling was captured using a *Zeiss AxioObserver.Z1 Apotome.2* with a temperature-controlled incubation chamber. Prior to imaging, immortalized 'chondrocyte-like' ATDC5 cells were stained with 5μM Fluo-8 AM (a green-fluorescent calcium indicator), washed in Hank's Balanced Salt Solution (HBSS), and immediately transferred to the microscope. For each condition, 5-minutes of baseline signaling collection occurred, followed by activation with DREADD ligands, TRPV4 agonist GSK101, 50% hypo-osmotic shock, or HBSS vehicle and imaging for a further 10-minutes. Imaging media, staining conditions, and imaging parameters—including light intensity, exposure time, and image intervals—were serially optimized to minimize fluorescence loss/photobleaching/phototoxicity across the 15-minute imaging sessions (in WT ATDC5 cells). Ca^{2+} signaling in response to DCZ and CNO were then assessed from 1pM to 100μM in both WT ATDC5 and ATDC5-hM3Dq-mCherry cells and compared against Ca^{2+} responses from GSK101 and osmotic shock. Individual cell Ca^{2+} responses were evaluated using a custom MATLAB program that extracts fluorescence intensity over time. To evaluate cell contractility, cells were stained with 9μM Hoechst 34580 (a blue nuclear/DNA stain) and imaged under both DIC transmitted light and UV/blue fluorescent light. Cells were imaged for two minutes to establish an un-activated baseline, then activated with 25nM CNO or DCZ and imaged continuously for 10 minutes to capture morphological changes and nuclear deformation after DREADD-activation.

Results: Modifying the staining media from HBSS + 0.04% Pluronic F-127 (manufacture's instructions) to 1:1 Dulbecco's Modified Eagle Medium/Ham's F12K without Pluronic and increasing incubation time from 30 minutes to 1 hour improved Fluo-8 AM uptake (less punctate intracellular staining) and overall cell health. Sequential optimization of imaging parameters (reducing light intensity, increasing exposure time, and increasing the time between image collection) as well as the addition of antioxidants (300μM Trolox [Vitamin E] and 500μM L-ascorbic acid [Vitamin C]) to imaging solution significantly improved fluorescent preservation (and reduced phototoxicity; seen as a reduction in both pycnotic nuclei and membrane blebbing and stable cell morphology) (Fig 1). Maintenance of a more consistent fluorescence intensity across live cell experiments improved our imaging of CNO- and DCZ-induced Ca^{2+} signaling through hM3Dq activation. DCZ induced similar Ca^{2+} oscillatory behaviors as CNO in the hM3Dq cells, while WT ATDC5 cells were unaffected by either DCZ or CNO (Fig 2). Finally, DIC imaging of cells revealed clear morphological changes within hM3Dq transfected cells directly after their activation with 500nM DCZ and CNO. Obvious nuclear deformations were observed following DREADD activation as well (Fig 3).

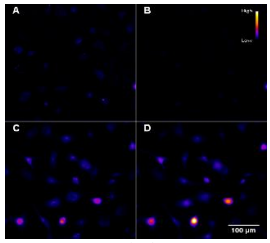

Figure 1: Heat map of Fluo-8 AM intensities in WT ATDC5 cells at the start (A&C) and end (B&D) of 15-minutes of imaging. A&B are for the original manufacture's protocol. C&D are for our loading and imaging protocol

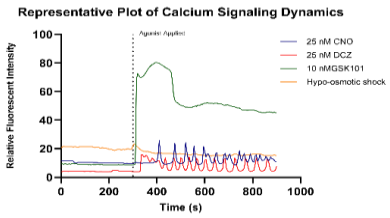

Figure 2: Representative Ca^{2+} traces for ATDC5-hM3Dq cells activated with the DREADD agonists CNO and DCZ (25 nM), 50% hypo-osmotic shock, or 10 nM GSK 101 (TRPV4 agonist)

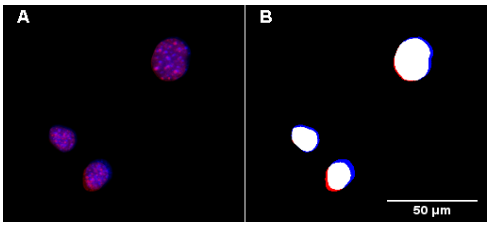

Figure 3: Nuclear deformations due to hM3Dq activation. **A)** ATDC5-hM3Dq-mCherry nuclei before (blue) and immediately after (red) 500nM CNO activation **B)** Visualization of nuclei regions before (red) & after activation (blue); white = nuclear regions that overlapped between pre- and post-activation.

Discussion: ATDC5 cells, when Fluo-8 AM is used to investigate Ca^{2+} signaling, appear especially susceptible to phototoxicity. While changes to dye loading and imaging conditions greatly improved cell health and fluorescent signal stability, additional improvements remain possible. Ongoing work will leverage high-speed spinning disk confocal microscopy to further mitigate photobleaching, while simultaneously allowing for video-rate imaging of Ca^{2+} signaling and cell responses following hM3Dq activation. Nevertheless, by improving our imaging capabilities, we show that both CNO and DCZ elicit oscillatory Ca^{2+} signaling in ATDC5-hM3Dq-mCherry cells; with DCZ serving as a promising—and safer—alternative to CNO use in murine models. Ongoing studies will expand on the initial morphological changes/cell contractions observed here following DREADD activation. We intend to employ traction force microscopy and intracellular tension sensors to measure cell contractility and force generation. Targeted inhibition of actin-myosin contractility will also be employed to investigate the role of Ca^{2+} oscillations and force generation on anabolic gene expression/neo-tissue formation.

Clinical Significance: Identifying the mechanisms linking oscillatory calcium signaling with anabolic gene expression outcomes will provide insight into the mechanisms that contribute to cartilage development/homeostasis and will provide new targets for tissue engineering/regeneration approaches to mitigate/reverse cartilage degradation associated with diseases such as osteoarthritis.