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HaloTag® Protein-Mediated Live Cell Imaging with Bigger FluoroNanogold™.

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The cardiac Na^{+}-Ca^{2+} exchanger, NCX1, plays a key role in multiple cardiac pathologies [1]. Electrophysiologically, an arrhythmogenic role has been proposed for NCX1 in cardiac failure [2] as asynchronous release of Ca^{2+} from intracellular stores leads NCX1 to depolarize myocytes at dangerous moments [3]. But perhaps the best defined pathological role of NCX1 is in ischemia-reperfusion injury in cardiac muscle. Studies conducted over past three decades have sown that Na^{+} loading of myocytes causes subsequent Ca^{2+} loading via Na^{+}/Ca^{2+} exchange. Moreover, Ca^{2+} load ultimately plays a major role in the damage incurred in ischemia-reperfusion [1,4]. Much of this damage can definitively be mitigated by blocking Na^{+} influx, which mostly occurs by Na^{+}/H^{+} exchange, [4,5] or by blocking Na^{+}/Ca^{2+} exchange [1], or both. Na^{+}/Ca^{2+} exchange knockout mice are protected nearly to the same extent achieved by classical cardiac preconditioning protocols [6]. Our preliminary biochemical and electrophysiological studies suggest that signaling pathways implicated in preconditioning cause a long-term reduction of NCX1 activity, perhaps via NCX1 internalization that is probably clathrin-independent [7,8]. In BHK or HEK293 cells, metabolic stress (such as glucose removal and Na^{+} loading by extracellular K^{+} removal) causes internalization of NCX1 (~80%) as determined by a recently developed PEGylation assay, in which two extracellular cysteines of NCX1 are labeled with PEG 5000-Maleimide to make localization NCX1 easier (PEG 5000 is used as a molecular weight reporter) [7]. The NCX1 membrane fraction decreases in parallel with an increase in phosphatidylinositol-(4,5)-bis-phosphate (PIP2) level (and vice versa); this has been described during circadian rhythms in entrained mice together with aortic banding, metabolic stress or over-expression of PIP2-synthesizing enzymes [7-9].

The reduction in activity of NCX1 during preconditioning can be attributed to its internalization. To verify this, it is desirable to be able to track NCX1 both in time in living cells and at ultrastructural resolution. To gain a more complete understanding of membrane trafficking of cardiac transporters, we have initiated a study employing optical methods for live cells [10] and state-of-the-art 3D ultrastructural method (focused ion beam/field emission scanning electron microscopy or FIB-FESEM) to localize NCX1 in fixed cells. For this purpose, a stable BHK (baby-hamster kidney fibroblast) cell line expressing NCX1 tagged with HaloTag® protein was developed. The HaloTag® is a mutant of bacterial dehalogenase (molecular weight ~34 kDa) that reacts with primary chloroalkanes (rate constant 10^{+6} $s^{-1}M^{-1}$) to form a stable covalent bond [11]. The cells were suspended in 20 mM phosphate buffer saline, pH 7.2, containing 2 mM Ca^{2+} and Mg^{2+} (PBS), and allowed to settle on a coverslip. They were then incubated with 5 µM HaloTag® Biotin ligand in PBS for 5 minutes and washed with PBS for 5 minutes. The biotin-labeled cells were incubated with a combined 1.8 nm gold and Alexa Fluor® 488-streptavidin FluroNanogold™ conjugate (1.5 µM in PBS) for 5 minutes, washed with PBS for 5 minutes and immediately imaged (**Figure 1 A**). In the control experiment, the HaloTag® Biotin ligand was omitted; as expected no membrane specific labeling was observed (**Figure 1 B**).

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The 1.8 nm gold nanoparticles were prepared by direct reduction of aqueous solution of Au(III) with sodium borohydride under basic conditions in the presence of proprietary thiols and conjugated to fluorescently-labeled streptavin following established protocols at Nanoprobes, Incorporated [12]. The combined 1.8 nm gold and Alexa Fluor® 488 labeled streptavidin FluoroNanogold™ conjugate contains about eight Alexa Fluor 488 and one 1.8 nm gold label per streptavidin molecule. The new bigger FluroNanogold™ can be used for multimodal imaging, such as fluorescence, bright-filed and electron microscopy, as previously described [13].

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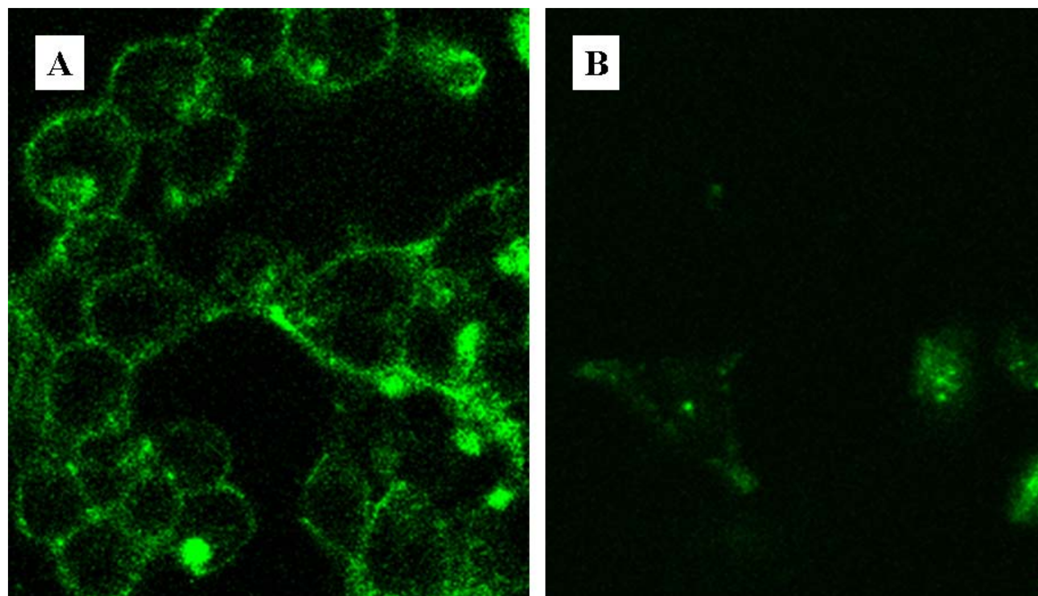

Figure 1. Fluorescence images of BHK cells expressing NCX1 surface protein fused to HaloTag® protein showing specific labeling with 1.8 nm gold-streptavidin-Alexa Fluor 488 FluoroNanogold™ (**A**) and control (**B**) when HaloTag® Biotin Ligand was omitted. Magnification 10X.

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