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**Fluorescent staining of sarcolemma in isolated cardiac myocytes - Challenges in research of t-tubule development**

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**Background:** Early stages of cardiac development in rats, characterized by appearance and growth of tubular system, may provide important clues in our understanding of adaptive processes occurring during cardiac hypertrophy. Since the cardiac myocytes of rats undergo rapid and extensive growth during the first month after birth, changes in cell morphology are accompanied by changes in the expression pattern of proteins involved in calcium signalling and by changes in excitation-contraction coupling.

**Purpose:** To study the functional consequences of T-tubule development and assembling of dyads during critical stages of rat myocardium growth, correlation between structural and functional parameters of isolated cardiomyocytes is necessary. Therefore we tested the use of fixable fluorescent membrane indicators in isolated cardiomyocytes. These would allow us to study localization and co-localization of the proteins involved in the formation, stability and function of dyads, with respect to their position relative to the sarcolemma.

**Methods:** The study followed the "Principles of laboratory animal care" (NIH Publication no. 85-23 revised 1985) and complied with the national law. Isolated cardiac myocytes of rats aged from 1 day to 8 weeks were stained with either fixable lipophilic dyes (FM 4-64 FX and mCling-ATTO 647N), or wheat germ agglutinin (a lectin used for staining proteins of the cellular membrane that contain N-acetylglucosamine and sialic acid residues) conjugated to Oregon Green 488 or Texas Red. After staining, cells were fixed with 4% formaldehyde (FM 4-68 FX and WGA conjugates) or with 4% formaldehyde + 0.2% glutaraldehyde (mCling-ATTO 647N). WGA conjugates were also used on cells pre-fixed with 4% formaldehyde. Cells were observed using the Leica TCS SP8 STED 3X confocal microscope with appropriate excitation/emission wavelengths.

**Results:** The lipophilic membrane indicators FM 4-64 FX and mCling-ATTO 647N provided a sufficient intensity of staining within 10 min of incubation at room temperature, and therefore were suitable for use on live isolated myocytes. After fixation, cells stained by FM 4-64 FX showed significant fluorescence of internal membrane systems. Fluorescent conjugates of wheat germ agglutinin required prolonged incubation at 37°C, up to 2 h in either live of pre-fixed cells, and therefore was not suitable for staining of live developing myocytes due to their high membrane dynamics. Selective staining of plasma membrane required a very high quality of myocyte isolation.

**Conclusions:** Although all three types of fluorescent dyes are highly selective indicators of the plasma membrane, their application in isolated cardiac myocytes requires specific changes to standard protocols, especially when working with myocytes in early stages of postnatal development.