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Freeze Substitution and Low Temperature Processing of Cryo Protected Tissues into Lowicryl HM23 Resin for Correlated Light and Immunogold Electron Microscopy in Drug Discovery and Safety Assessment Research

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Correlative light and electron microscopy (CLEM) and immunogold electron microscopy (iEM) are important methods in the arsenal of experimental strategies applied in the field of drug discovery and safety assessment of potential therapeutic molecules. The precise localization of drug targets and drug candidates in cultured cells, organoids and mammalian tissues plays a major role in elucidating the mechanism of action of a drug candidate and to better understand safety concerns and pathologies associated with potential therapeutic molecules.

Cryo-fixation by plunge freezing or high pressure freezing followed by freeze-substitution and low temperature processing of samples into Lowicryl resins [1] has generally been recognized as one of the best sample preparation strategies for CLEM and iEM, because it has the potential to better preserve ultrastructural detail, membrane contrast, epitopes and fluorescence emission compared to conventional methods [2]. Cryo-fixation however is only reliable for thin samples (below 0.4 mm) and is not suitable for most mammalian tissues and organoids without first thin-sectioning by vibratomy, which can be challenging when regions of interest and histopathologies are ill defined or very small and focal.

Here we present an alternative processing strategy which promises to be more efficient and suitable for CLEM and iEM applications in drug research and safety assessment that often involve large study groups (triplicates of control and treatment groups) of mammalian tissues. We combined perfusion fixation with cryo-protection of harvested organs in dimethylsulfoxide (DMSO) to reliably freeze and process larger tissue blocks (several mm^3 in volume). During freeze-substitution in acetone (at -80^0C) we minimized the amount of uranyl acetate (0% to 0.01%) to preserve heavy metal sensitive epitopes and fluorescence emission. Tissues were then processed into HM23 resin at -80^0C to reduce extraction of lipids and other biomolecules and to better preserve ultrastructural detail (instead of using the more popular HM20 resin at -40^0C). Semi-thin sections of UV-polymerized tissue blocks were then placed on carbon-coated glass slides, contrasted with osmium tetroxide, uranyl acetate and lead citrate and imaged by wide-field backscattered electron scanning EM with or without immunogold labeling.

With this procedure we consistently achieved well preserved ultrastructure in various tissues, such as mouse colon (Figure 1, A-C), colorectal tumors (Figure 1, D-E), spleen, pancreas and tonsils (not shown). Furthermore, proteins of interest, such as alpha-synuclein, were detected and localized through the preservation of fluorescent tags like GFP, or by using anti-synuclein antibodies conjugated to both fluorophores and colloidal gold particles (Figure 1, F). We are currently applying this promising strategy to localize drug targets, novel antibiotics and therapeutic nanoparticles in various animal tissues.

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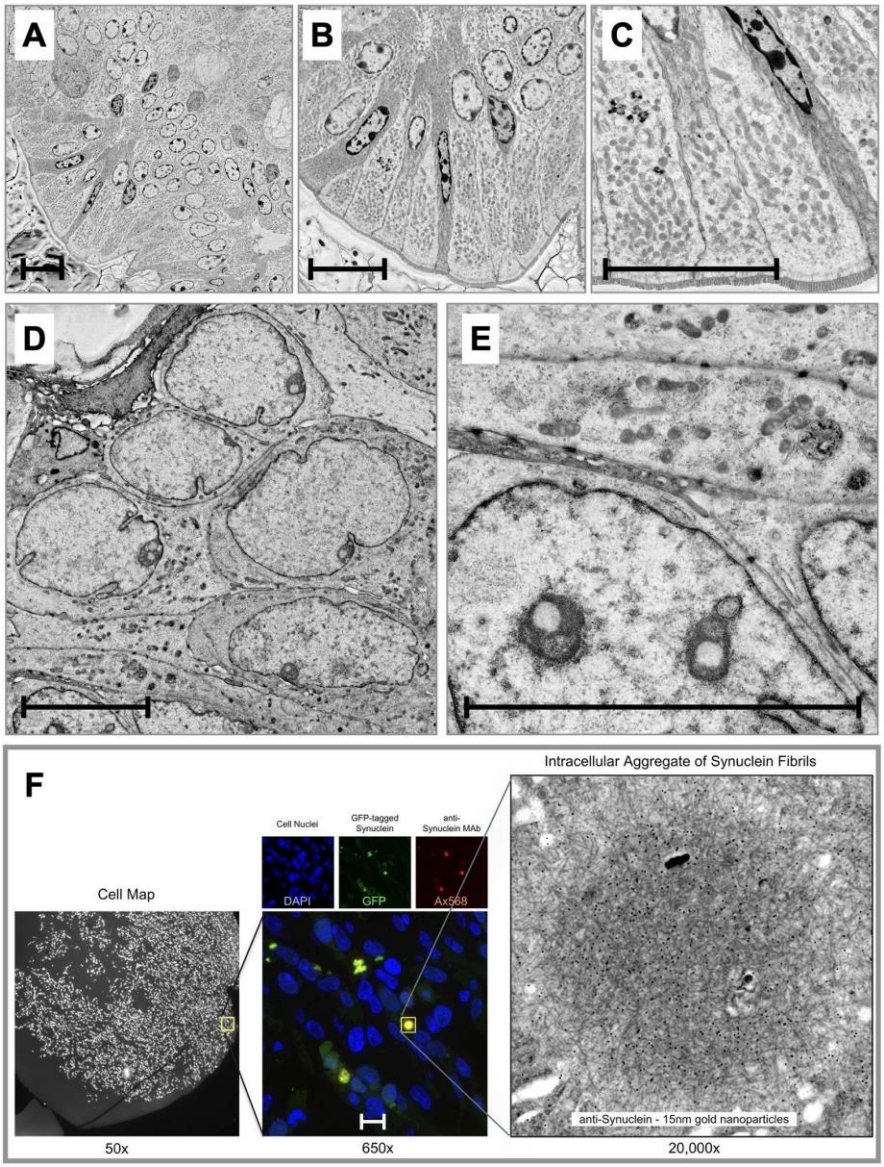

Figure 1. Preservation of cryo-protected tissues and cells after freeze-substitution and low temperature processing into Lowicryl HM23 resin for CLEM and immunogold EM applications. (A-C) Epithelial cells in mouse colon tissue at various scales. (D and E) Tumor cells in mouse colorectal cancer tissue. (F) CLEM imaging of gfp-synuclein expressing cells. Overview cell map (left image, gray scale), immunofluorescence imaging (middle panel) with nuclei (DAPI, blue), gfp-synuclein (green) and anti-synuclein labeling (red); and correlated anti-synuclein immunogold EM (15 nm gold particles) of a large synuclein inclusion (right panel). Scale bars are 10 micrometer.

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