**Core G subcellular fractionation of RAW 264.7 cells**

Alex Andreyev 5/12/2008

36 x $10^6$ RAW264.7 cells were plated in 24 ml of complete Lipid MAPS medium in T-150 flask. At 24 hrs after plating they were treated with 100 ng/ml $KDO_2$-Lipid A (or vehicle control) for another 24 hrs followed by subcellular fractionation. Briefly, for fractionation the cells were harvested by scraping, washed with PBS and isolation medium (250 mM sucrose, 10 mM HEPES-KOH, pH 7.4, 1 mM EGTA) and pelleted in hypotonic medium (same as above but with only 100 mM sucrose). Cell pellet was homogenized in the hypotonic medium by 40 strokes in Dounce homogenizer and subjected to differential centrifugation in the isolation medium (supplemented with 2 mM $MgCl_2$): 200 g to pellet nuclei/unbroken cells („nuclear" pellet), 5,000 g to pellet mitochondria and 100,000 g to pellet microsomes. Postnuclear and postmitochondrial supernatants were additionally spun to remove residual nuclei and mitochondria, respectively. Mitochondria were additionally washed to remove contaminating microsomes/ER and $Mg^{2+}$. Supernatant from 100,000 g spin was retained as cytosolic fraction. Nuclear, mitochondrial, and microsomal pellets were additionally separated in stepwise gradient of iodixanol (Optiprep, Sigma). Nuclei banded at the 30%/35% iodixanol interface in 20 min 10,000 g spin in $Mg^{2+}$-containing medium. Mitochondria, plasma membrane, and ER banded at the 17.5%/25%, 10%/17.5% and 17.5%/25% interfaces, respectively, in 2 hr 50,000 g spin in $Mg^{2+}$-free medium.. All samples were frozen and stored at -80ºC.

Purity of the fractions was characterized with regard to intensities of specific markers for each organelle/cell compartment (DNA for nuclei, succinate dehydrogenase for mitochondria, NADPH-dependent cytochrome c reductase for ER, K-dependent phosphatase for plasma membrane and glucose 6-phospate dehydrogenase for cytosol).