



LipidTOX™ Dyes for Adipocyte Staining in Routine Imaging Applications

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Abstract

Models for adipogenesis have been a tool for mapping regulatory pathways. The fluorescent dye Nile red is commonly used to identify adipocytes, but is not an ideal choice as it labels both phospholipids and neutral lipids and has a broad emission spectrum, rendering multiparametric assays difficult. We examined three novel dyes, LipidTOX™ Green, Red, and Deep Red Neutral Lipid Stains, for lipid droplet staining after 3T3-L1 and human mesenchymal stem cell differentiation into adipocytes. Our data show that the LipidTOX stains are specific for neutral lipids and have narrower emission spectra than Nile red, enabling multiparametric assays. We observed brighter signal and lower background with LipidTOX stains in comparison to Nile red. We further show multiplexing of LipidTOX dyes with a FABP4 antibody. Overall, LipidTOX neutral lipid stains showed superior performance over Nile red, defining these new tools as the more selective and flexible reagents for applications in adipogenesis and pathway mapping.

Introduction

LipidTOX™ stains have been developed by Molecular Probes™ Invitrogen Labeling and Detection Technologies for staining cellular lipids for HCS applications. The purpose of this study was to describe the application of LipidTOX™ Neutral Lipid Stains as a replacement for Nile red in a standard non-HCS fluorescence microscopy platform, and to use it for monitoring differentiation of mouse 3T3-L1 fibroblasts and human mesenchymal stem cells into adipocytes. We also show a correlation of LipidTOX Green stain in lipid droplets and the expression of a protein associated with adipogenesis, Fatty Acid Binding Protein 4 (FABP4), in adipocytes from induced 3T3-L1 fibroblasts.

Materials & Methods

Mouse 3T3-L1 Fibroblast Culture and Differentiation

3T3-L1 fibroblasts (ATCC) were incubated in DMEM + 10% FBS (Gibco®) at 37°C in 5% CO₂ until they reached 70% confluency. They were seeded on coverslips at P3 and adipogenesis was induced by the MDI method, using 1 µM dexamethasone, 0.5mM IBMX, and 10 µg/ml insulin (MacDougald 1994). The cells were fixed for staining on day 10 after induction. Un-induced control 3T3-L1 fibroblasts on coverslips were maintained in DMEM + 10% FBS (Gibco®) until harvesting, with media changes every two days.

3T3-L1 Neutral Lipid Staining

Coverslips were rinsed in PBS, then fixed in 1% formaldehyde and washed in PBS. They were incubated with either LipidTOX (Invitrogen) at 1:200 in PBS or with Nile red, 500 nM in PBS, for 30 minutes. Afterwards they were stained briefly with 300 nM DAPI, rinsed in PBS, and mounted on glass slides with Prolong® Gold mounting media (Invitrogen).

Materials & Methods

3T3-L1 Staining with LipidTOX Neutral Lipid Stain and FABP4 antibody

Coverslips with induced or uninduced 3T3-L1 fibroblasts were fixed in formaldehyde/PBS fixative, then permeabilized in wash buffer containing 0.1% saponin (Sigma) in PBS. They were incubated in a blocking solution containing 10% normal goat serum (Invitrogen) in wash buffer for 30 minutes, followed by rabbit anti-FABP4 IgG (Cayman Chemical), 4 µg/ml in blocking solution, for 1 hour. The coverslips were washed in wash buffer, then incubated with goat anti-rabbit Alexa Fluor™ 594 IgG (Invitrogen), 2 µg/ml in blocking solution, for 1 hour. The coverslips were washed in PBS then stained with LipidTOX Green stain and DAPI as described in previous section.

Human Mesenchymal Stem Cell (MSC) Culture

MSCs were obtained from fresh human bone marrow aspirates, expanded for two passages, harvested, frozen and banked in liquid nitrogen. Vials of frozen MSCs were thawed and incubated in T-162 flasks with DMEM low glucose (GIBCO®) + 10% MSC-qualified FBS (GIBCO®) + 4 mM L-glutamine + 50 mg/ml gentamicin (GIBCO®) at 37°C, 5% CO₂. Culture media was replaced with fresh media one day after thaw, sub-cultured every 4 - 5 days at 80-90% confluency, and replated at 3 - 4 x 10³ cells/cm². MSCs were passaged no more than four times post-freeze (six passages in all) before additional banked MSCs were retrieved for further studies.

Differentiation

MSCs were harvested with TryPLE™ (GIBCO®) and plated at 2 x 10⁴ cells/cm² in 96-well plates. Cells were allowed to attach for two hours and media was replaced with adipogenic differentiation media (DMEM low glucose, 10% MSC-qualified FBS, 2 mM GlutaMAX™, 50 mg/ml gentamicin, 58 µg/ml bovine insulin, 1 µM dexamethasone, 200 µM indomethacin, 500 µM IBMX) and cultured for a period of 9 days. To assay for the presence of differentiated adipocytes, the cultures were fixed and stained with 0.5% Oil Red O in 60% isopropanol solution.

MSC Neutral Lipid Staining

The cells were rinsed in PBS, fixed in formaldehyde/PBS fixative, then washed in PBS and stained for 30 minutes either with LipidTOX Green, Red or Deep Red Neutral Lipid stains at 1:125 dilution in PBS, or with Nile red 3 µM in PBS.

Figure 1 – Detection of Adipogenesis in Differentiated 3T3-L1 fibroblasts with LipidTOX Neutral Lipid Stains

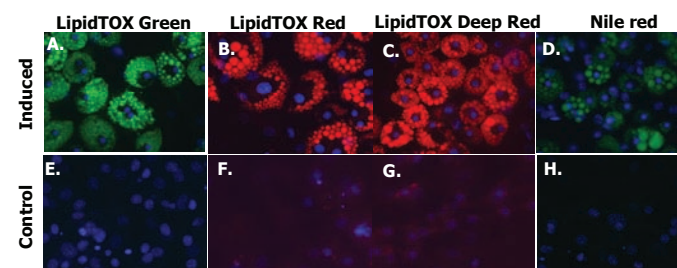


Fig. 1A-H Comparison of LipidTOX stains and Nile red in 3T3-L1 fibroblasts. Images were taken at 40X magnification on a Nikon E800 fluorescence microscope, captured monochromatically, and computer colorized before combining without further digital processing. Adipocytes from induced 3T3-L1 fibroblasts (A-D) show lipid droplets. These droplets are absent in uninduced control cells (E-H). 1A & E) LipidTOX™ Green stain, FITC filter, lipid droplets = green, DAPI stained nuclei = blue. 1B & F) LipidTOX Red stain, TRITC filter, lipid droplets = red. 1C & G) LipidTOX Deep Red stain, TRITC filter, lipid droplets = red. 1D & H) Nile red, FITC filter, lipid droplets = green. Nile red showed lower signal and higher background than LipidTOX stains.

Figure 2 – Detection of Adipogenesis in Differentiated Mesenchymal Stem Cells with LipidTOX Neutral Lipid Stains

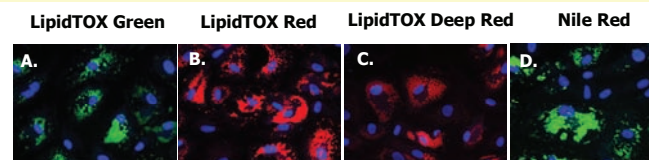


Fig. 2 LipidTOX stain and Nile red in adipocytes from differentiated human MSCs, 40X magnification. Cells were imaged on a Nikon TE300 inverted microscope, captured monochromatically, and digitally processed to enhance color before combining. A) LipidTOX Green stain, FITC filter, lipid droplets = green. B) LipidTOX Red, TRITC filter, lipid droplets = red. C) LipidTOX Deep Red, TRITC filter, lipid droplets = red. D) Nile red, FITC filter, lipid droplets = green.

Figure 3 – Correlation of LipidTOX Green Stain in Lipid Droplets with FABP4 Expression in Adipocytes

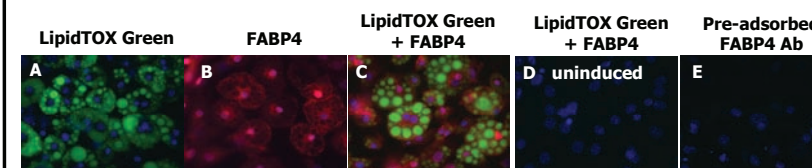


Fig. 3 Adipocytes from differentiated 3T3-L1 fibroblasts, 40X magnification A) LipidTOX Green stain, FITC filter, lipid droplets = green, DAPI stained nuclei = blue. B) Rabbit anti-FABP4 labeled with goat anti-rabbit Alexa Fluor™ 594 IgG (red) on induced adipocytes, TRITC filter. C) Adipocytes stained with both LipidTOX Green stain (green) and anti-FABP4 IgG (red). D) LipidTOX Green stain and Anti-FABP4 IgG, uninduced 3T3-L1 fibroblasts. No lipid droplets or FABP4 protein observed in control uninduced 3T3-L1 cells. E) Pre-adsorbed antibody alone, on induced adipocytes. Antibody was pre-adsorbed with FABP4 peptide, aa103-118, (Cayman Chemical) before use, as a check for antibody specificity.

Figure 4 – LipidTOX Green Stain vs Nile Red

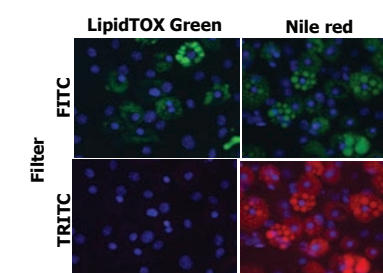


Fig. 4 Lipid droplet staining in differentiated 3T3-L1 fibroblasts, same slides imaged with two filters. LipidTOX Green shows lipid droplets (green) only with the FITC filter, compared to Nile red which shows droplet staining (green and red) in both FITC and TRITC filters.

Results and Conclusions

- LipidTOX Neutral Lipid stains have several advantages over Nile red for adipogenesis assays, including specificity for neutral lipids, greater signal intensity, and lower background levels.
- Nile red staining for lipid droplets was detected with both FITC and TRITC filters, which limits the ability to run multiparametric assays.
- The emission spectra for Nile Red lipid staining is dependent on hydrophobicity, with phospholipids emitting in the TRITC filter range and neutral lipids emitting in FITC range. However, Nile red staining of neutral lipid droplets was detected in both FITC and TRITC filter ranges (Fig. 4), suggesting that the reported emission specificity of Nile red may not be reliable. (Greenspan, 1985).
- LipidTOX stains are an effective replacement for Nile red and provide flexibility for assays. Cell fixation is not required (data not shown), suggesting they could also be useful tools for live cell microscopy and flow cytometry.

References

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