

Oil red-O stains non-adipogenic cells: a precautionary note

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Abstract

Bovine adipofibroblasts, 3T3-L1 cells, L-6 myogenic cells, and sheep satellite cells were allowed to proliferate for 48 h. Oil red-O (ORO) was dissolved in three different solvents isopropanol, propylene glycol and triethyl phosphate. At 48 h, the proliferative cultures were stained with the three stains. ORO stain prepared in both propylene glycol and triethyl phosphate resulted in bright red droplets appearing in all cultures, whereas ORO dissolved in isopropanol was not taken up by any of the cells. These data suggest that certain preparations of ORO may stain cells in non-adipogenic lineages as well as undifferentiated pre-adipocytes. Caution must be exercised when choosing solvents for ORO in differentiation studies using cells of the fat/adipose lineage.

Abbreviations: DMEM – Dulbecco's modified Eagle's medium; FBS – Fetal bovine serum; ORO – Oil red-O; PBS – Phosphate-buffered saline, pH 7.08; PEG – Propylene glycol; PSG – Pig skin gelatin; SC – Satellite cell; TEP – Triethyl phosphate

Introduction

It is generally acknowledged that proliferation and differentiation are diametrically opposite events in cell biology. Consequently, a fibroblast-like adipofibroblast (or preadipocyte) cell residing in its proliferative state, and which has not entered into the terminal event of differentiation, is not thought to be capable of accumulating lipid. Alternatively, once the adipofibroblast accumulates lipid, it is traditionally considered to be an adipocyte. Cell

staining is often used to identify cell morphology, as well as the developmental compartment in which the cell resides. For example, oil red-O (ORO) staining has facilitated knowledge regarding the differentiation state of adipofibroblasts, as they physiologically convert from proliferative-competent cells into differentiated (lipid filled) adipocytes (Vierck et al. 1996), and in the study of lipogenesis (Vierck et al. 2001). Under light microscopy, adipocyte-derived lipid droplets that are stained with ORO appear bright red/orange, leaving the

remainder of the cellular constituents non-stained (nuclei may be counter-stained). Isopropanol (Lillie and Ashburn 1943; Ramirez-Zacarias et al. 1992; Fukumoto and Fumimoto 2002), propylene glycol (PEG) (Putt 1972) and triethyl phosphate (TEP) (Koopman et al. 2001) have all been used as solvent-carriers for ORO.

Recently, our laboratory has been expanding beef cattle-derived adipofibroblasts, to evaluate cell morphology during early differentiative events, and to verify the regulation required to effect complete cell differentiation (Adebonojo 1974; Johnson and Francendese 1985). An end-point of this research was to devise a defined medium that would induce adipofibroblasts to differentiate into lipid-containing multilocular adipocytes within 48 h. The staining data of Figure 1 suggest that lipid may accumulate in differentiated bovine adipocytes (and parallel cultures of 3T3-L1 cells used as a control/reference cell line) 48 h after plating, as documented by staining with ORO dissolved in either isopropanol (a–b), PEG (c–d), or TEP (e–f). However, during many of our most recent studies with bovine adipofibroblasts, we observed that proliferating cultures of a variety of cells were capable of assimilating ORO, if certain solvents were employed (Lillie and Ashburn 1943; Putt 1972; Feldman and Dapson 1974; Ramirez-Zacarias 1992; Koopman et al. 2001; Fukumoto and Fumimoto 2002). Therefore, we designed a series of validation experiments to investigate the specificity and staining properties of ORO when dissolved in three different solvents.

Materials and methods

Cell lines

Bovine adipofibroblasts were isolated from the subcutaneous fat depot of an adult steer, expanded in culture, and stored in liquid nitrogen (Vierck et al. 1996). Parallel cultures of 3T3-L1 cells (ATCC CL173) and negative control L-6 myogenic cells were purchased from the American Type Culture Collection and stored in liquid nitrogen. In addition to the use of L-6 myogenic cells, callipyge sheep satellite cells (SC) were isolated from the semimembranosus muscle, expanded in culture, stored in liquid nitrogen

(Dodson et al. 1986), and used in the present study. Both L-6 myogenic cells and sheep SC do not accumulate lipid during proliferation or differentiation.

Media and reagents

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen/Life Technologies, 12800-017) prepared by dissolving 13.5 g of DMEM and 3.7 g NaHCO₃ (Sigma, S-5761) in 900 ml of glass-distilled H₂O. The pH was adjusted to 7.08 and brought to volume in a 1000 ml volumetric flask. The medium was sterile filtered in a laminar flow hood (Nu Aire, NU-425-4000) by passing the medium through a 0.22 μm pore filter into two sterile 500 ml glass bottles and stored at 4 °C. In a laminar flow hood, the DMEM was supplemented with 10% fetal bovine serum (FBS) (Invitrogen/Life Technologies, 26140-079), 1% of penicillin–streptomycin (Invitrogen/Life Technologies, 15140-122) and 0.5% of gentamicin (Invitrogen/Life Technologies, 15710-064). Phosphate-buffered saline (PBS) was prepared by dissolving 10.0 g NaCl (Sigma, S-5886), 0.25 g KCl (Sigma P-5405), 1.44 g Na₂HPO₄ (Sigma, S-5761) and 0.25 g KH₂PO₄ (Sigma, P-5655) in 900 ml of glass-distilled H₂O. The pH was adjusted to 7.08 and then brought to volume using a 1000 ml volumetric flask. The solution was then aliquoted into two 500 ml glass bottles and autoclaved for 30 min at 121 °C at 15 psi. The PBS/EDTA (ethylenediamine tetraacetic acid) trypsin solution was prepared by adding 0.168 g Na₂EDTA · 2H₂O (Invitrogen/Life Technologies, 15576-028) to 1000 ml of PBS, then aliquoting into four 250 ml glass bottles and sterilizing by autoclaving for 30 min at 121 °C at 15 psi. A 25 ml aliquot of sterile trypsin (Sigma, T-4549) was then added to 225 ml of sterile PBS/EDTA and stored at 20 °C. Pig skin gelatin (PSG) (Sigma, G-1890) was prepared by adding 0.5 g of PSG to 400 ml glass-distilled H₂O in a glass beaker and heating to (80 °C) on a heating plate. The solution was then brought to volume in a 500 ml volumetric flask, aliquoted into five 100 ml glass bottles, autoclaved for 30 min at 121 °C at 15 psi and then stored at 4 °C. A solution of 10% buffered neutral formalin was prepared by adding 10 ml of

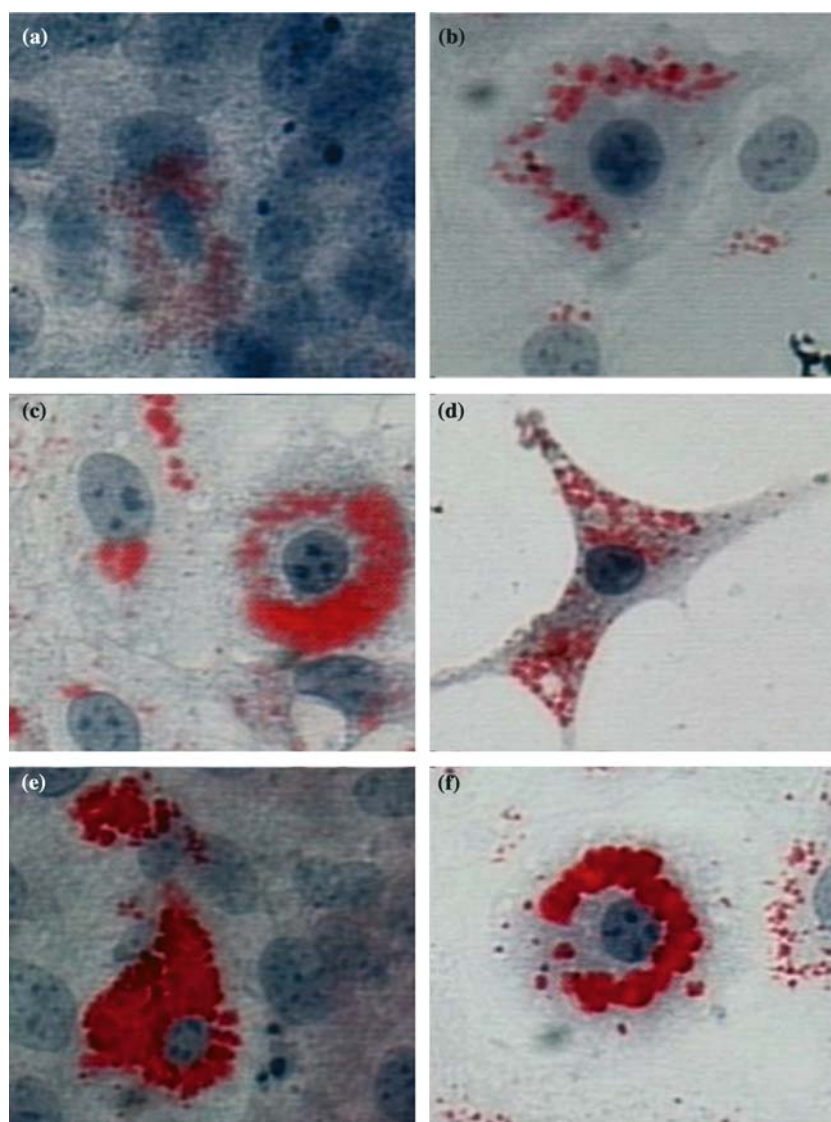


Figure 1. Differentiated bovine adipocytes (derived from bovine adipofibroblasts) and 3T3-L1 cells stained with ORO dissolved in three different solvents. (a) Differentiated bovine adipocytes (400 \times) and (b) 3T3-L1 cells (400 \times) stained with ORO/isopropanol. The lipid droplets appear as red/orange, and both cell types showed a halo/horseshoe shape around the nuclei. (c) Bovine adipocytes (400 \times) and (d) 3T3-L1 cells (400 \times) stained with ORO/propylene. The lipid droplets appear orange/red. A halo was evident around the nuclei of the 3T3-L1. (e) Bovine adipocytes (400 \times) and (f) 3T3-L1 cells (400 \times) stained with ORO/triethyl phosphate. The lipid droplets in both sets of cells appear bright red. Additionally, bovine adipocytes appear to have smaller lipid droplets making up the formation of the lipid halo around the nucleus. The 3T3-L1 cells possessed larger lipid droplets.

formaldehyde (JT Baker, 2106-01) to 90 ml PBS. Sodium phosphate was prepared by dissolving 1.0 g Na_2PO_4 (Sigma, S-5136) in 100 ml glass-distilled H_2O and was stored at room temperature (22 $^\circ\text{C}$). A 60% solution of isopropanol was prepared by adding 40 ml glass-distilled H_2O to 60 ml of 100% 2-propanol (Fisher Scientific, A416-4).

Cell culture

The bovine adipofibroblasts, 3T3-L1 cells, L-6 myogenic cells and sheep SC were thawed and suspended separately in 20 ml of DMEM + 10% FBS. Each cell type was then centrifuged (2700 rpm for 3 min; 1500 $\times g$) using a Beckman TJ-6

centrifuge (horizontal rotor) and resuspended in 20 ml DMEM + 10% FBS. Callipyge sheep SC and L-6 myogenic cells were plated in PSG-coated 75 cm² flasks (Corning Incorporated Life Sciences, 430725) and the bovine adipofibroblasts and 3T3-L1 cells were plated in uncoated 75 cm² flasks. All cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air for 24 h, then the DMEM + 10% FBS was removed and fresh DMEM + 10% FBS was added. When the cells were approximately 75% confluent (usually 48–72 h), they were detached from the culture dishes with trypsin/EDTA, centrifuged at 2700 rpm (1500 × g) for 3 min and resuspended in 20 ml DMEM + 10% FBS. Bovine adipofibroblasts, Callipyge sheep SC, 3T3-L1 cells and L-6 myogenic cells were counted on the Coulter (particle) Counter (Beckman Coulter, Z1) and then replated on PSG-coated (Callipyge sheep SC and L-6 myogenic cells) or non-coated (bovine adipofibroblasts and 3T3-L1 cells) 24-well plates (Nalge NuncTM, 143982) (5000 cells/well) or on Lab-Tek II 8-well chamber slides (Nalge NuncTM, 154534) (1000 cells/well). The cells were grown in parallel using DMEM + 10% FBS for a period of 24–48 h (Gamou et al. 1990) and incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air for 24 h. At 24 h, the DMEM + 10% FBS was removed, and fresh DMEM + 10% FBS was added. At 48 h, the plates and slides were removed from the incubator and prepared for staining.

Staining

ORO/isopropanol method (Lillie and Ashburn 1943; Ramirez-Zacarias et al. 1992; Fukumoto and Fumimoto 2002; Figure 2)

ORO (1.4 g; Sigma, O-0625) was dissolved in 400 ml of 99% 2-propanol and left over night at room temperature (22 °C). The solution was next filtered using Whatman #4 filter paper (Whatman International, 1004 185), mixed with 144 ml glass-distilled H₂O and left over night at 4 °C. The solution was then filtered again using Whatman #4 filter paper, left to stand for 30 min, and filtered (Whatman #4) again before use. Before staining, the media was removed from the wells with a Pasteur pipette or by gently inverting the plate over a waste container, then gently rinsing with PBS. One milliliter of 10% formalin was

added to each well for 10 min to fix the cells. Each well was then rinsed with 60% isopropanol for 30 s. ORO stain was applied 1 ml per well for 10 min and removed, and then the wells were rinsed with 60% isopropanol for 5 s. Next, the wells were rinsed with glass-distilled H₂O for approximately 1 min followed by the addition of 1 ml per well of Mayer's hematoxylin solution (Sigma, MHS-32) for 10 min. The cells were then washed with glass-distilled H₂O, and sodium phosphate solution (ml per well) was added for 5 min to intensify the staining of the nuclei. The wells were then rinsed twice for 30 s with glass-distilled H₂O, air dried and mounted with two drops of Crystal/MountTM (Biomedica Corp, M02), a water-based preservative.

ORO/PEG method (Putt 1972; Figure 2)

ORO (0.5 g) was dissolved in 10 ml of 100% PEG (Fisher Scientific, P355-1). An additional 90 ml of PEG was added gradually while heating the solution to 95 °C. The solution was then filtered with a coarse filter paper (Whatman #4), left for 14 h at room temperature (22 °C) and again filtered (Whatman #4) before use. The media was then removed from the wells with a Pasteur pipette or by gently inverting the plate over a waste container. The wells were gently rinsed with PBS, and the cells were fixed for 10 min with 1 ml per well of 10% formalin. After fixing, the wells were rinsed with glass-distilled H₂O three times for 30 s per rinse. Next, 1 ml of 100% PEG was added to each well for 2 min and then removed with a Pasteur pipette or by gently inverting the plate over a waste container. ORO/PEG (1 ml per well) was added, allowed to remain for 15 min, removed and then the wells were rinsed in 60% PEG for 1 min. The wells were rinsed in glass-distilled H₂O, and 1 ml per well hematoxylin was added for 10 min. The wells were then washed with glass-distilled H₂O three times for 30 s each. The staining of the nuclei was intensified with 1 ml per well of sodium phosphate for 5 min, followed by a glass-distilled H₂O rinse for 30 s. The wells were air dried and mounted with two drops of a water-based preservative (Crystal/MountTM).

ORO/TEP method (Koopman et al. 2001; Figure 2)

ORO/TEP stock was prepared by dissolving 500 mg ORO in 60 ml of 99% TEP (Sigma, T-61107) and 40 ml glass-distilled H₂O. Prior to

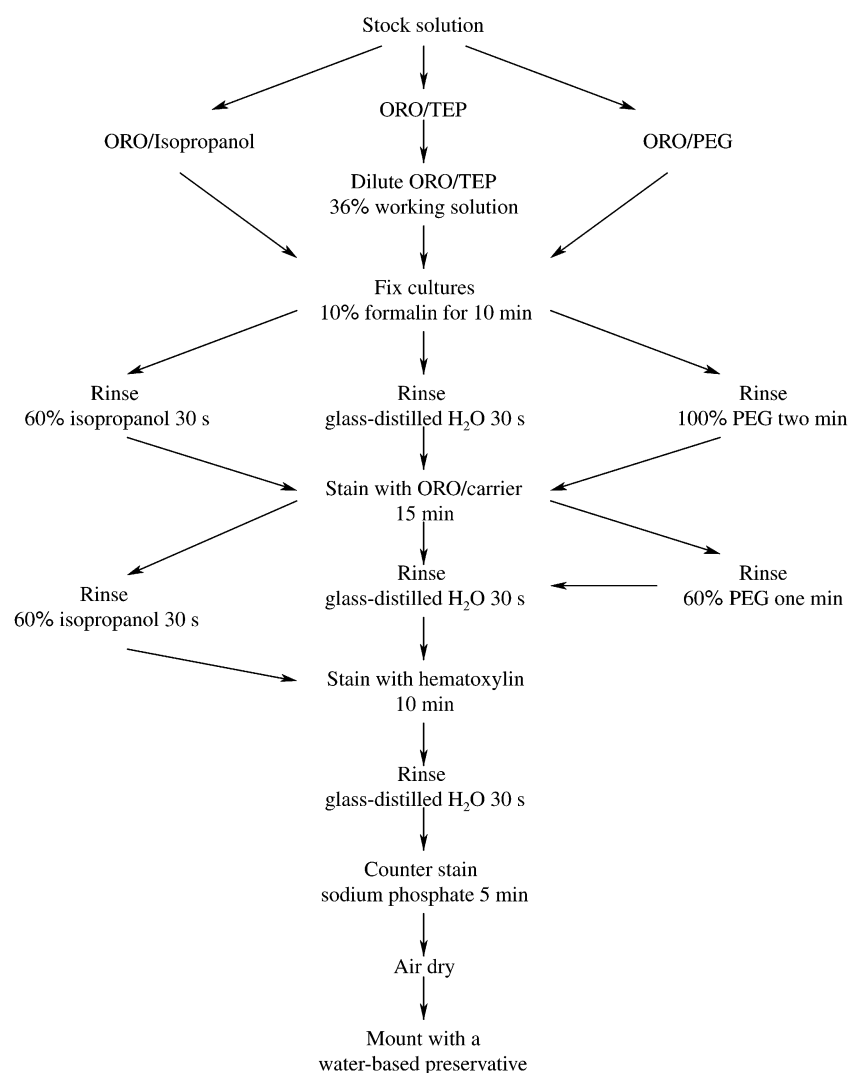


Figure 2. Flow diagram summarizing three ORO staining procedures used in cell culture.

use, 12 ml of the stock was diluted with 8 ml of glass-distilled H₂O to yield a 36% ORO/TEP working solution. The medium was removed from the wells with a Pasteur pipette or by gently inverting the plate over a waste container, and the wells were gently rinsed with PBS. The cells were fixed for 10 min with 1 ml per well of 10% formalin. After fixing, the wells were rinsed with glass-distilled H₂O three times for 30 s each. ORO/TEP was applied (1 ml per well) for 15 min followed by a gentle rinse three times with glass-distilled H₂O for 1 min. Hematoxylin (1 ml per well) was added for 10 min to counterstain the nuclei, followed by one more rinse in glass-distilled H₂O

for 1 min. The staining of the nuclei was enhanced by adding 1 ml per well sodium phosphate solution for 5 min and rinsing with glass-distilled H₂O for 1 min. The wells were allowed to air dry and then mounted with two drops of a water-based preservative (Crystal/MountTM).

Analysis

The staining effectiveness was evaluated with a Nikon TMD Diaphot (Nikon) inverted research grade microscope equipped with a Sony CCD/RGB color video camera (0.6 inch chip)/Optics

image analysis system. Images (100× or 400×) were captured and enhanced using Image-Pro® Plus (Media Cybernetics®), version 4.0 for Windows™.

Results and discussion

Each of the four types of cells, bovine adipofibroblasts, 3T3-L1 cells, L-6 myogenic cells, and sheep SC, were plated for 48 h to allow the cells to attach and proliferate. Once the cells were actively proliferating, they were stained and evaluated for

lipid content. The data contained in Figures 3a–b suggest that proliferating bovine adipofibroblasts, stained with ORO/isopropanol, did not take up the stain in the cytoplasm around the nucleus. However, in parallel adipofibroblast cultures, approximately 70% of the cells stained with ORO/PEG possessed cytoplasmic stain, which was visualized as small perinuclear droplets in a halo shape (Figures 3c–d). Adipofibroblast cultures stained with ORO/TEP displayed red droplets within the cytoplasm, which were observed as small perinuclear droplets forming a horseshoe or halo (Figures 3e–f).

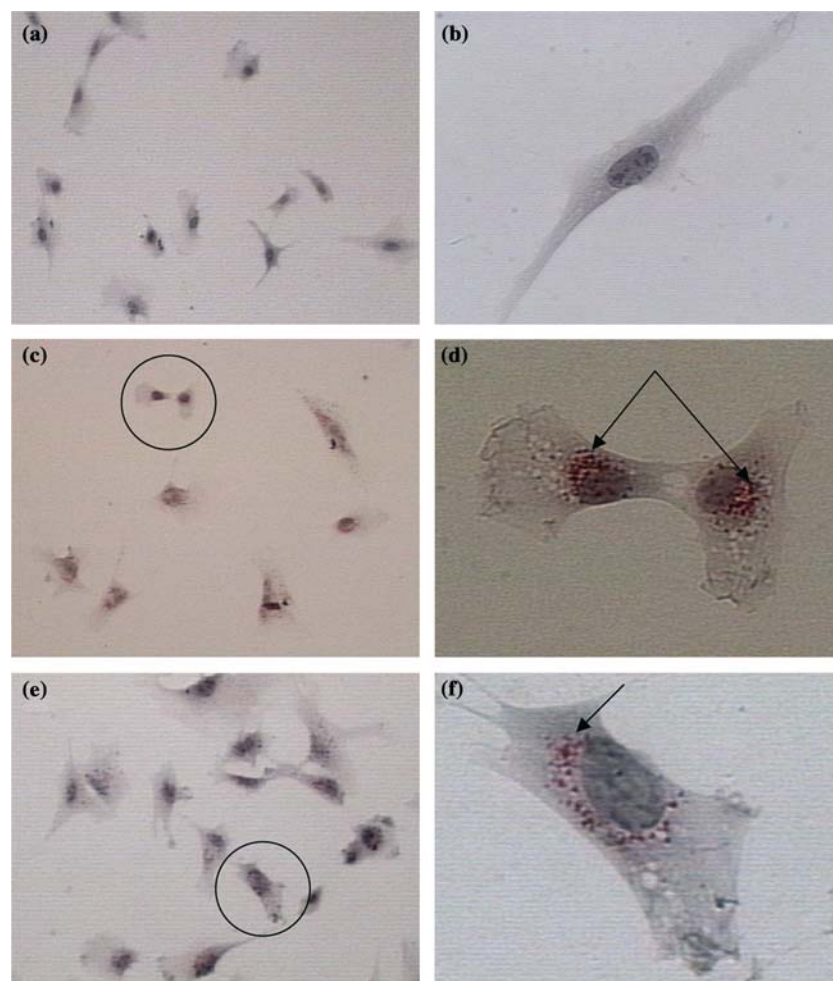


Figure 3. Proliferative bovine adipofibroblasts stained with ORO dissolved in three types of solvents. (a) Cells stained with oil red-O/isopropanol (100×) show no uptake of stain; similar results occur with the cell in b (400×). (c) Cells stained with ORO/propylene glycol (100×) and (e) ORO/triethyl phosphate (100×) appears to absorb the stain in a perinuclear fashion. The black circles indicate the insets d (ORO/propylene glycol) and f (ORO/triethyl phosphate) both at 400×. The arrows point toward round lipid-like droplets around the nuclei that absorbed the stains.

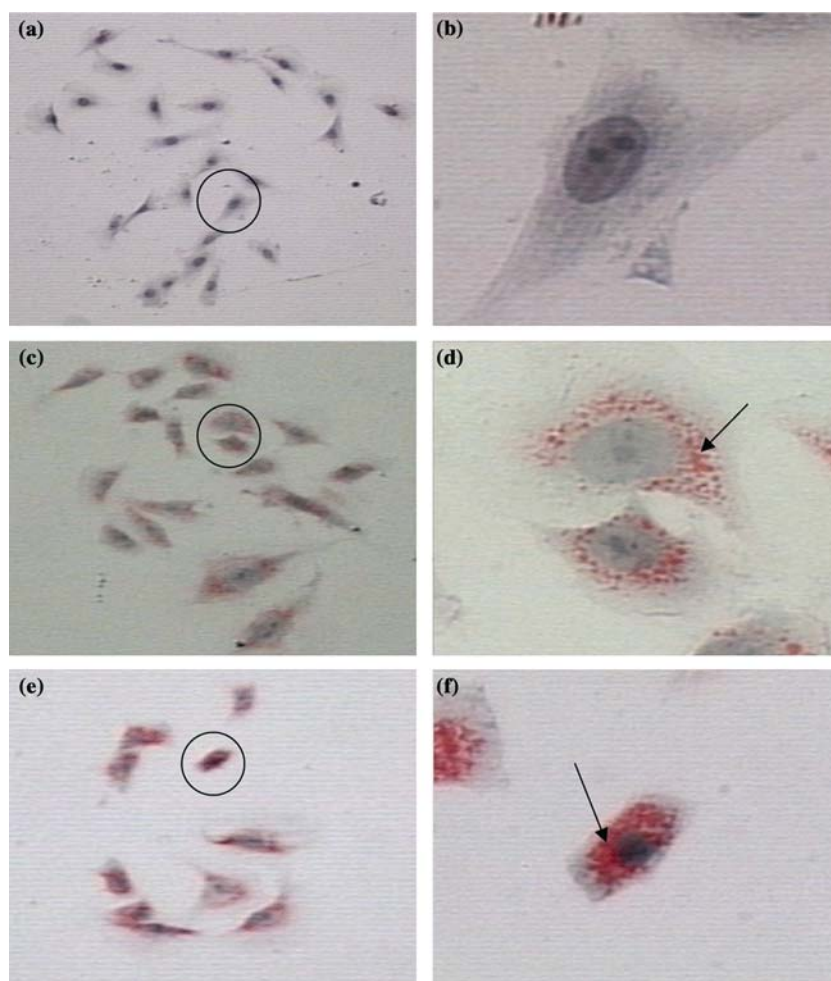


Figure 4. Proliferative L-6 myogenic satellite cells stained with ORO dissolved in three types of solvents. (a) The cells stained with ORO/isopropanol (100 \times) show no uptake of stain. (b) A 400 \times view of the circled area in (a) validates the lack of stain uptake. (c) Cells stained with ORO/propylene glycol (100 \times) and (e) ORO/triethyl phosphate (100 \times) show the uptake of stain. Represented by the black circles in (c) and (e); (d and f) at 400 \times show the round lipid-like droplets around the nuclei (black arrows).

Proliferating 3T3-L1 cells were used as a positive parallel comparison with the bovine adipofibroblasts and produced the same results as described previously. There was no uptake of stain seen in 48 h cultures with the use of ORO/isopropanol (data not shown). A large percentage of the cells stained with ORO/PEG also displayed small, orange/red lipid-like droplets around the nuclei. Conversely, there was 100% uptake of the ORO/TEP stain (seen as small, bright red lipid-like droplets in the shape of a horseshoe or halo around the nuclei of the cells).

Observation of the staining of the negative control cultures of L-6 myogenic cells with ORO/

isopropanol (Figures 4a–b) did not indicate that the cells assimilated any stain. However, cultures of L-6 myogenic cells possessed the same small lipid-like droplets in the perinuclear region when stained with ORO/PEG (Figures 4c–d) or ORO/TEP (Figure 4e–f). Similar to the bovine adipofibroblast and 3T3-L1 cell cultures, 100% of the L-6 myogenic cells assimilated ORO/TEP stain, and 70% of the L-6 myogenic cell cultures stained with ORO/PEG.

Sheep myogenic SC cultures stained with ORO/isopropanol were negative for stain uptake as expected, but cultures exposed to both ORO/PEG and ORO/TEP showed signs of stain uptake

(data not shown). With both ORO/PEG and ORO/TEP treatments the stain was found in small lipid-like vesicles contained within the cytoplasm; however the droplets were more random and did not display the tight horseshoe or halo shape around the nuclei as did the bovine adipofibroblasts, 3T3-L1 cells, and L-6 myogenic cells.

In summary, parallel cultures of proliferating bovine adipofibroblasts, 3T3-L1 cells, L-6 myogenic cells, and sheep SC, showed no stain uptake while using ORO/isopropanol. However, in the adipofibroblast cultures, ORO/TEP and ORP/PEG stains resulted in red/orange staining of lipid-like structures appearing around the nuclei. In the myogenic SC the red/orange stain was more dispersed intracellularly. It is unclear at this time exactly what is being stained in the cells when using ORO/TEP or ORO/PEG, although it is possible that the carriers are binding to other structures within the cell. Further research must be conducted to determine what structures these ORO stains are visualizing. Regardless, we recommend that caution be taken when trying to determine amounts of differentiation in adipocyte cell cultures while using TEP or PEG as a carrier for ORO.

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References

- Adebonojo F.O. 1975. Studies on human adipose cells in culture: relation of cell size and cell multiplication to donor age. *Yale J. Biol. Med.* 48: 9–16.
- Dodson M.V., McFarland D.C., Martin E.L. and Brannon M.A. 1986. Isolation of satellite cells from ovine skeletal muscles. *J. Tissue Cult. Meth.* 10: 233–237.
- Feldman A. and Dapson R. 1974. Relative effectiveness of various solvents for oil red O. *Med. Lab. Tech.* 31: 335–341.
- Fukumoto S. and Fumimoto T. 2002. Deformation of lipid droplets in fixed samples. *Histochem. Cell Biol.* 118: 423–428.
- Gamou S., Shimizu Y. and Shimizu N. 1990. Adipocytes. In: Pollard J.W. and Walker J.M. (eds), *Methods in Molecular Biology*, Vol. 5. Humana Press, New Jersey, pp. 197–207.
- Johnson P.R. and Francendese A.A. 1985. Cellular regulation of adipose tissue growth. *J. Anim. Sci.* 61(Suppl 2): 57–75.
- Koopman R., Schaart G. and Hesselink M.K.C. 2001. Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochem. Cell Biol.* 116: 63–68.
- Lillie R.D. and Ashburn L.L. 1943. Supersaturated solutions of fat stains in dilute isopropanol for demonstration of acute fatty degeneration not shown by Herxheimer's technique. *Arch. Pathol.* 36: 432–440.
- Putt F.A. 1972. *Manual of Histopathological Staining Methods*. John Wiley, New York, pp. 194–196.
- Ramirez-Zacarias J.L., Castro-Munozledo F. and Kuri-Haruch W. 1992. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with oil red O. *Histochem. J.* 97: 493–497.
- Vierck J.L., Dal Porto D. and Dodson M.V. 2001. Induction of preadipocyte differentiation by a defined treatment medium without DMI. *Basic Appl. Myol.* 11: 99–104.
- Vierck J.L., McNamara J.P. and Dodson M.V. 1996. Two alternative procedures to isolate adipofibroblasts from sheep skeletal muscle. *Meth. Cell Sci.* 18: 309–314.