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methods

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Abstract Interest in measuring tissue lipids has increased as the link between fat-laden tissues and metabolic disease has become obvious; however, linking disease to a specific cell type within a tissue has been hampered by methodological limitations. Flow cytometry (FC) has been used to assess relative lipid levels in cells. Unfortunately, its usefulness is limited because comparisons between samples generated over several hours is problematic. We show that: *1*) in lipophilic fluorophore stained cells, fluorescence intensity measured by FC reflects lipid levels; *2*) this technique can be used to assess lipid levels in a mixed cell population; *3*) normalizing to a control condition can decrease experiment-to-experiment variation; and *4*) fluorescence intensity increases linearly with lipid levels. This allows triacylglycerol (TG) mass to be estimated in mixed cell populations comparing cells with known fluorescence and TG levels. We exploited this strategy to estimate lipid levels in monocytes within a mixed population of cells isolated from human blood. Using this strategy, we also confirmed that perilipin (PLIN)1 increases TG accumulation by ectopically expressing fluorescently tagged PLIN1 in Huh7 cells. In both examples, biochemically assaying for TG in specific cell populations is problematic due to limited cell numbers and isolation challenges. Other advantages are discussed.—Wolins, N. E., K. N. DeHaan, V. Cifarelli, and A. K. Stoeckman. **Normalized neutral lipid quantitation by flow cytometry. *J. Lipid Res.* 2018.** 59: **1294–1300.**

Supplementary key words lipid droplet • BODIPY® 493/503 • monocytes • triacylglycerol • perilipin

With industrialization came greater food availability, longer life span, and the decreased need for physical activity. There has been a subsequent increase in adiposity, tissue steatosis, and metabolic disease (1–3). Obesity has long been associated with an increased prevalence of metabolic disease and recent studies reveal an even stronger association between liver steatosis and metabolic disease (4, 5). A more detailed understanding of lipid deposition likely will reveal even more specific associations with disease. Unfortunately, data on lipid levels in specific cell types within tissues is scarce. This is in part due to limited options to assess lipid levels in specific cells in tissues composed of multiple cell types.

Imaging has been used, but has significant disadvantages. First, it is either subjective or requires laborious schemes to quantify lipids. Second, heterogeneity within a sample and the requirement for high magnification to see small lipid droplets makes data collection tedious. Third, even with schemes to quantify signals between samples, the absolute amount of lipids per cell is difficult to estimate. Flow cytometry (FC) does not share these disadvantages; it is automated and quantitative, is not subject to observer bias, and does not analyze a microscopic part of a heterogeneous tissue. Not surprisingly, FC has been used to assess lipid levels in mixed populations of cells (6). However, the usefulness of FC is diminished because, like quantitative PCR and many other assays, units are arbitrary and differ between instruments and experiments. Further, unlike many biochemical assays, which can be performed on banked frozen samples, FC is generally performed on live cells. Finally, whereas most other biologic assays have strategies to normalize measurements between experiments, no such normalization has been devised for FC lipid measurements. These constraints limit the usefulness of FC in any study requiring measurements before and after treatment. We show here that fluorescence intensity measured by FC increases linearly with triacylglycerol (TG) levels in cells, that normalizing to a control condition can improve comparisons between experiments, and that fluorescence intensity of a single cell type in a mixed population can be assessed.

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Abbreviations: BD, BODIPY® 493/503; BDMFI, BODIPY® 493/503-dependent mean fluorescence intensity; eGFP, enhanced green fluorescent protein; FC, flow cytometry; FSC, forward scatter; PBMC, peripheral blood mononuclear cell; PLIN, perilipin; RFP, red fluorescent protein; SSC, side scatter; TG, triacylglycerol.

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MATERIALS AND METHODS

Cell culture and FA treatment

AML-12 mouse hepatocytes were cultured in DMEM/F12 containing 10% FBS, antibiotics, and 0.1% Insulin-Transferrin-Selenium (Thermo Fisher 41400-045). HEK293 and Huh7 cells were cultured in DMEM containing 10% FBS and antibiotics. U937 cells were cultured in RPMI-1640 with 10% FBS and antibiotics. The FA, oleate, was complexed with FA-free BSA at 8:1 or 5.5:1 molar ratio, as indicated in the figure legends (7, 8).

Flow cytometry

Cells were treated as described in the figure legends. At the time of harvest, cells were trypsinized and washed once with PBS, followed by staining with either the live cell method or the fixed cell method.

Live cell method. Cells were stained with 1 ml PBS containing 10 μg of BODIPY® 493/503 (BD) (Thermo Fisher D3922) for 15 min at room temperature. Cells were then washed once with PBS before being analyzed on a Partec CyFlow Space ML flow cytometer.

Fixed cell method. Cells were trypsinized and fixed with 4% formaldehyde for 20 min at room temperature. Cells were stained with 20 ng/ml BD for 30 min, washed three times with PBS, and analyzed using an LSR Fortessa cytometer (BD Bioscience) and FlowJo software (FlowJo LLC, Ashland, OR).

Fluorescence microscopy

Fluorescence microscopy was performed as described previously (7). In brief, 3.5×10^5 cells were plated onto 22 mm square glass coverslips in 6-well tissue culture plates containing growth medium supplemented with FA. After 24 h, the medium was removed and cells were fixed in 3% paraformaldehyde. Fixed cells were washed four times with PBS and then 2 ml of microscopy buffer with 20 μl of 1 mg/ml BD were added to each well for 1 h at room temperature in the dark. Cells on coverslips were again washed four times with PBS followed by mounting to slides with ProLong® Gold antifade reagent with DAPI. Slides were imaged with a Nikon Eclipse TE2000-U microscope. Images were captured with a Photometrics CoolSnap cf camera using MetaMorph software (Molecular Devices, Downingtown, PA).

Triacylglycerol assay

An aliquot of the cells grown under each condition was assayed for TG. Cells were trypsinized, washed once with PBS, resuspended in PBS with 10 mM EDTA, and then counted. Cells were divided in triplicate into 13 × 100 mm glass tubes in a final volume of 200 μl. Triolein standards (Sigma T7140) were also prepared in a final volume of 200 μl PBS/10 mM EDTA in 13 × 100 mm glass tubes. TGs were extracted and quantified as previously described (9). Briefly, 2 ml of isopropanol:hexane:water (40:10:1) were added to cells or standards and the samples were vortexed, covered, and incubated at room temperature for 30 min. Then, 500 μl of a 1:1 mixture of hexane:diethylether were added to the samples followed by vortexing and incubating for an additional 10 min at room temperature. Next, 1 ml of water was added to samples, tubes were vortexed, and layers were allowed to separate at room temperature while covered for 30–45 min. Using Pasteur pipettes, the top layer was transferred to 12 × 75 mm glass tubes and dried under N_2 to completion. Following the drying step, 400 μl of Infinity triglyceride reagent (Thermo Scientific TR22421) were added to each tube and vortexed. Tubes were covered and incubated for 90 min at 37°C with shaking at 250 rpm. Finally, 300 μl of each sample were transferred to 96-well plates and absorbance was measured at 540 nm using a microplate reader. To determine whether fixing cells affected the TG measurement, we quantified TG in fixed and unfixed cells from the same batch of Huh7 cells incubated in 2 mM FA. We measured 159 ± 10.7 pg/cell (n = 6) and 160 ± 3.37 pg/cell (n = 5) in the unfixed and fixed cells ($P = 0.80$), respectively.

Peripheral blood mononuclear cell isolation

Blood was drawn and collected in BD Vacutainer Lithium Heparin tubes (Thermo Fisher 02-689-7). Freshly isolated blood was diluted with an equal volume of PBS and layered on a Ficoll-Paque™ PLUS cushion according to the manufacturer's instructions (GE Healthcare 17-1440-02). After a 40 min spin at 400 *g* (no brake), peripheral blood mononuclear cells (PBMCs) were collected, washed, and incubated with the following anti-human antibody cocktail: CD45 (1:200), HLA-DR (1:200), CD11c (1:1,000), and CD14 (1:60) (all from Biolegend, San Diego, CA) for 20 min at 4°C. Cells were then washed, fixed in BD Cytofix buffer (BD Bioscience), and stained with 20 ng/ml BD for 30 min at room temperature (fixed cell method). Cells were analyzed with an LSR Fortessa cytometer. These experiments were approved by the Bethel Institutional Review Board or monitored by the Human Research Protection Office at Washington University School of Medicine in St. Louis, MO (clinicaltrials.gov number: NCT01538836).

Red fluorescent protein-perilipin 1 fusion construct

The red fluorescent protein (RFP)-perilipin (PLIN)1 expression construct was made by replacing the sequence encoding enhanced green fluorescent protein (eGFP) with that of monomeric RFP (10) in the previously described peGFP-C2-PLIN1 construct (11). The sequences linking the vector to RFP-PLIN1 are cgctagcgctaccggtcgccaccATG and aag agc TGAaagcttcgaa and the sequence that links RFP to PLIN1 is cac ctg ttc ctg gag / atc tca ata aac. The start and stop sites are capitalized, "/" shows the junction between the RFP and PLIN1, and spaces in the sequence indicate codons.

RFP-PLIN1 transfection

Huh7 cells were grown to 60–80% confluence and then transfected with RFP-PLIN1 using 7 μg RFP-PLIN1 in 1.2 ml Opti-MEM (Thermo Fisher 11668027) and 30 μl of Lipofectamine 2000 in 1.2 ml Opti-MEM (Thermo Fisher 31985062). Opti-MEM containing the DNA and Lipofectamine were combined, incubated for 10 min, and then added to cells (400 μl/well to a 6-well plate or 2.4 ml to a 100 mm plate). Transfection medium was replaced after 6 h with medium containing 100 μM FA.

RESULTS

Clinicians and physiologists work with tissues composed of multiple cell types. Traditionally, lipid levels were assessed biochemically in the whole tissue or in specific cell types by microscopy using lipophilic dyes. Microscopy and FC measure arbitrary fluorescent units, which vary with the day and between instruments. This makes comparisons between experiments performed at separate times problematic. We sought to develop a FC-based method to assay relative lipid levels in specific cell types within a mixed cell population, which does not require samples to be assayed in parallel for comparison.

FC assesses relative lipid levels of a single cell type within a mixed population

The first goal required that relative lipid levels of a single cell type be assessable in a mixed cell population. To

generate cells with different TG levels, we cultured kidney cells (HEK293) in 100 μM FA and hepatocytes (AML-12) in 400 μM FA. As expected, fluorescence microscopy showed that AML-12 cells contained more and larger TG droplets than HEK293 cells (**Fig. 1A**). We then mixed these cells and assessed size and granularity by forward scatter (FSC) and side scatter (SSC), respectively. We gated intact cells away from debris (large gate Fig. 1B). Within this gate, we identified two SSC-resolvable cell populations (Fig. 1B, upper left). We then analyzed the incorporation of a lipophilic dye by assessing BD fluorescence in the FITC channel and observed two distinct peaks (Fig. 1B, upper right). When gated separately, the cell population with increased SSC (internal complexity likely due to increased TG droplets) had a mean BD-dependent mean fluorescence intensity (BDMFI) of 34.7 (Fig. 1B, lower right), approximately 5-fold higher than the other cell population (BDMFI = 7.3; Fig. 1B, lower left). To confirm the identity of the cell populations, HEK293 cells and AML-12 cells were analyzed individually and BDMFIs were determined at 8.9 and 36.5, respectively (data not shown). As expected, the AML-12 cells cultured with a higher concentration of FA had increased SSC and BDMFI.

Normalizing data between experiments

Comparing to cells with known lipid levels. Lipid loading increased cell lipid droplet number and BDMFI (Fig. 1). To determine whether there is a quantitative relationship between TG and BDMFI, we generated cells with different TG levels by varying the FA concentration in their media and then measuring their average mass of TG/cell and BDMFI. **Figure 2** shows that a linear relationship exists between TG mass and BDMFI within a cell type, regardless of whether the cells were analyzed live (Fig. 2A) or after fixation (Fig. 2B). Further, **Fig. 3A** shows that BDMFI increased at a similar rate independent of cell type [Huh7 slope = 51 ± 6 (fg/cell)/BDMFI and U937 slope = 49 ± 7 (fg/cell)/BDMFI]. However, non-TG-dependent BDMFI (the intercepts) appeared to vary between cell types. Linear regression indicated that the TG-independent BDMFI

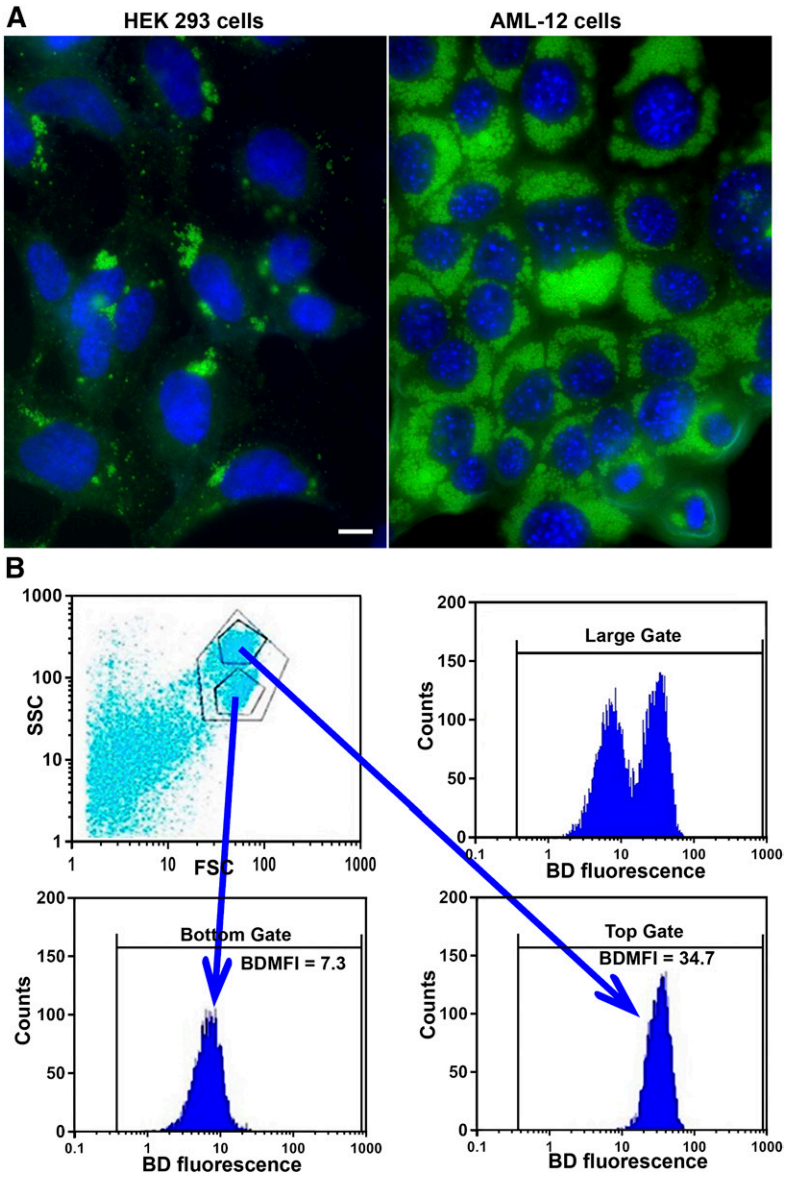

Fig. 1. FC can resolve a mixed cell population into cells with high lipid levels or low lipid levels. A: HEK293 cells and AML-12 cells were cultured overnight in growth medium containing 100 μM FA or 400 μM FA, respectively (complexed 8:1 with BSA). BD staining is shown in green and DNA staining by DAPI is shown in blue. Scale bar, 10 μM. B: One million HEK293 cells and one million AML-12 cells were cultured overnight in growth medium containing 100 μM or 400 μM FA (complexed 8:1 with BSA), respectively. Cells were stained with BD using the live cell method and then mixed. BDMFI was determined by FC. Results shown are representative of three experiments.

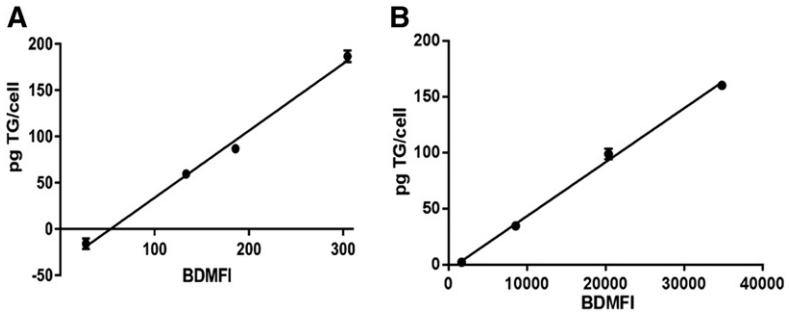

Fig. 2. BD fluorescence increases linearly with lipid levels. A: AML-12 cells were cultured overnight in growth medium supplemented with 0, 100, 200, or 400 μM FA (complexed 8:1 with BSA) and were assayed for BDMFI or TG using the live cell method. B: U937 cells were cultured for 72 h in growth medium supplemented with 0, 100, 300, or 1,000 μM FA (complexed 8:1 with BSA) and then fixed. TG was assayed and BDMFI determined using the fixed cell method. $R^2 > 0.99$ for both data sets. The negative TG value shown in panel A denotes that the absorbance in the TG assay for AML-12 cells was less than the standards with no TG. This result likely is due to a compound extracted from the AML-12 cells interfering with enzyme-linked dye reductions in the TG assay.

(x-intercepts) is 1,082 for Huh7 cells and 1,891 for U937 cells. In fact, we have found multiple orders of magnitude difference in BDMFI between different types of lean cells (data not shown). The observation that there was a consistent lipid-dependent increase in BDMFI indicates that lipid levels can be estimated by interpolation using cells with known BDMFI and lipid levels. However, as shown in Fig. 3A, cell-types differ in lipid-independent BDMFI (BDMFI at x-intercept). This necessitates that the cells used to generate the standard curve have similar lipid-independent BDMFI as the cells being assessed.

Standardizing to a specific condition. Normalizing BDMFI to a specific treatment condition can be advantageous. This mitigates lipid-independent differences in mean fluorescence intensity (e.g., staining conditions, instrument type, and settings). The utility of this scheme can be seen by comparing Fig. 3B (non-normalized BDMFI) with Fig. 3C. In Fig. 3C, data from separate experiments was normalized by dividing the BDMFI of cells grown at each level of supplemented FA by the BDMFI of cells grown without supplemental FA (the lipid-independent BDMFI). Therefore, this scheme is more precise than interpolating between cells with known TG levels. However, this scheme cannot be used to estimate an absolute lipid mass and even relative amounts between samples cannot be estimated without knowing the TG-independent BDMFI.

Applications

We used the linear relationship between lipid and BDMFI to estimate TG levels in mixed cell populations. First, the monocyte cell line, U937, was used as a standard to estimate TG in monocytes within human PBMCs (**Fig. 4**). We gated $CD14^+$ cells within the CD11c and HLA-DR double positive PBMC population to identify monocytes and then determined the BDMFI. In parallel, we determined the BDMFI in U937 monocytes with known amounts of TG. We fitted BDMFI and TG of the U937 monocytes to a line and used this line to estimate that these human monocytes had 1.94 pg TG/cell.

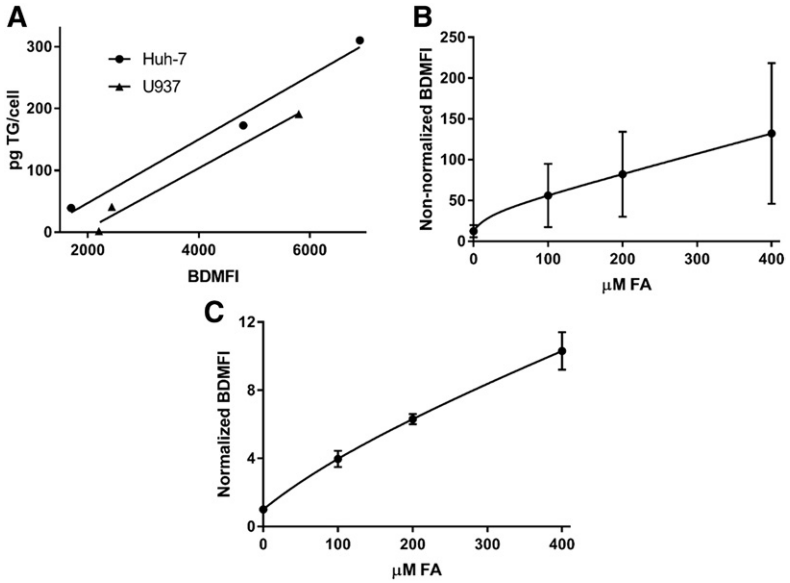

Fig. 3. Normalized lipid levels. A: Huh7 and U937 cells were cultured overnight in growth medium containing 0, 100, and 1,000 μM FA (5.5:1, FA:BSA) and cells were then fixed. TG was measured and BDMFI was assessed using the fixed cell method. B: AML-12 cells were incubated with the concentration of FA (8:1, FA:BSA) indicated and BDMFI was measured using the live cell method (n = 3). C: Data from panel B was normalized by dividing the BDMFI at each FA concentration by the BDMFI of the cells grown with 0 μM FA.

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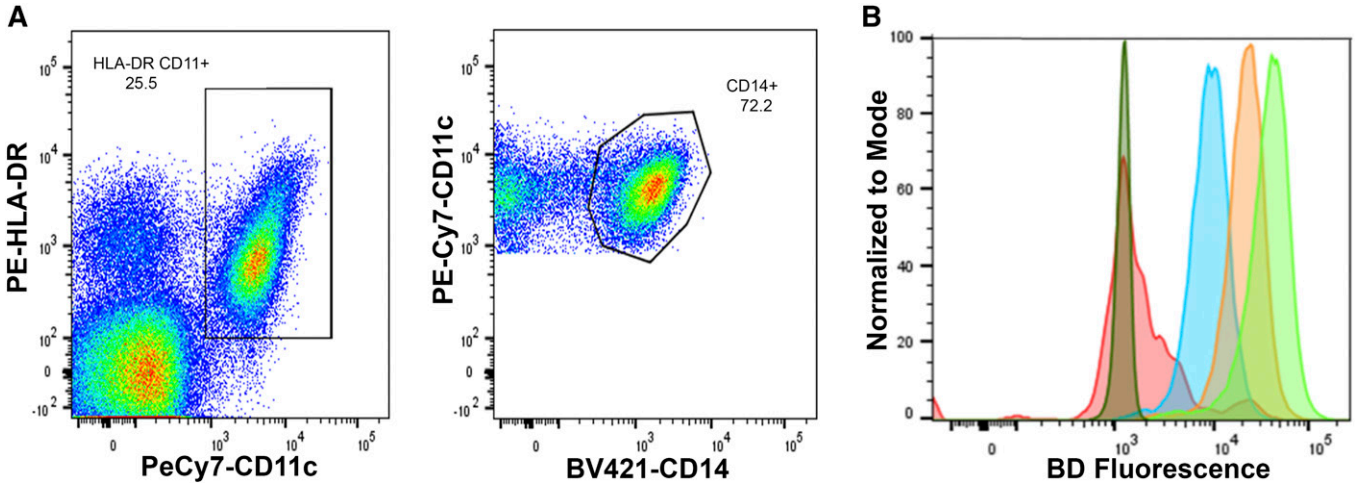

Fig. 4. FC can be used to estimate TG in a mixed cell population. A: Human PBMCs were isolated and fixed as described in the Materials and Methods. These cells were stained and analyzed in parallel with the U937 cells shown in Fig. 2B. Blood monocytes were identified as $CD14^+$ cells gated from the $CD45^+CD11c^+HLA\text{-}DR^+$ population. Then, the BDMFI was measured in the $CD14^+$ population. B: The U937 cells that are described in Fig. 2B comprise the red, blue, orange, and light green peaks and have 2.13, 34.7, 98.8, and 160.1 pg TG/cell, respectively. The darker green peak represents the blood monocytes.

Second, we used nontransfected Huh7 cells with known amounts of TG to estimate the TG in Huh7 cells transfected with RFP-PLIN1. PLIN1 increases TG levels by inhibiting its degradation (12).The RFP signal was used to identify the cells transfected with this construct (**Fig. 5A**). As previously reported for PLIN1 expression, we found that RFP-PLIN1 expression increased TG accumulation 10-fold (12) (Fig. 5B, C). However, within the RFP-PLIN1 transfected population, when the best fit line of Huh7 cell standards was used to estimate TG mass, the increase in RFP-PLIN1 mean fluorescence intensity did not correlate with an increase in TG levels (Fig. 5C). In fact, the low and moderate levels of expression (gates 2–5) drove the most TG accumulation and the highest level was least effective (gate 6). PLIN1 is well-studied and this lack of dose dependence has not been reported in cell systems. Hence, we confirmed this observation by imaging BD-stained RFP-PLIN1 cells transfected in parallel with those analyzed by FC (Fig. 5D). Because *1*) this image lacked cells with exceptionally strong RFP signal, *2*) only 30 cells were shown, and *3*) only a few percent of cells were in gate 6, we surmise that RFP-PLIN1 levels comparable to gate 6 levels are not likely shown. Consistent with the FC data and previous reports, we observed that transfected Huh7 cells had more BD staining than those that escaped transfection. Further, in the transfected cells (labeled L, M, and H), RFP-PLIN1 dose did not correlate with lipid accumulation as assessed by BD signal (Fig. 5D). Interestingly, both PLIN1 ablation and its overexpression in mice diminish their adipocyte TG stores (13–15). This is consistent with our FC-based observation that compared with cells with no PLIN1 or very high PLIN1 levels, cells with intermediate levels of PLIN1 have large TG stores.

DISCUSSION

We show here that the propensity of lipophilic fluorochrome BD to partition into lipid can be used to assess intracellular lipid levels. Because this partitioning is an intrinsic property of BD, it works on fixed or living cells and under a wide range of concentrations. BDMFI can be normalized using cells with known lipid mass (Fig. 2) or incubated under specific conditions (Fig. 3), allowing meaningful comparisons between experiments (Figs. 4, 5). We show that using cells with known TG levels as standards allows per-cell TG mass to be estimated, and we propose that this can be done in any gatable cell population where there is a cell type with a comparable amount of nonlipid BD fluorescence. This makes estimates of lipid mass in rare cell types in a complex tissue feasible. Normalizing by using cells with known per-cell lipid masses allows the additional advantage of being able to make meaningful comparisons between independent studies.

These FC strategies have several advantages over previous methods. Unlike biochemical assays, FC does not require an organic extraction, which is tedious and increases measurement error. Additionally, biochemical assays are prone to interference from non-acyl glycerol in the samples, as discussed in the report detailing the biochemical assay (9), and is demonstrated by the negative value for TG in Fig. 2A. Also, this method is more quantitative, more objective, and less laborious than imaging strategies. Finally, because data is collected for each cell, cell-to-cell variation in lipid levels is determined. As an example, U937 monocytes have more variations in BDMFI than monocytes from human circulation. This likely is due to the U937 monocytes being propagated extensively outside the quality control mechanisms that maintain the homogeneity of circulating monocytes.

Other signals can be determined simultaneously with BD giving context to lipid estimates. These include SSC, FSC, and a vast and growing number of fluorescently tagged lipids, proteins, and antibodies and stains (filipin, phalloidin, and DAPI). Further, because FC measures these signals in each cell, the relationship between lipid

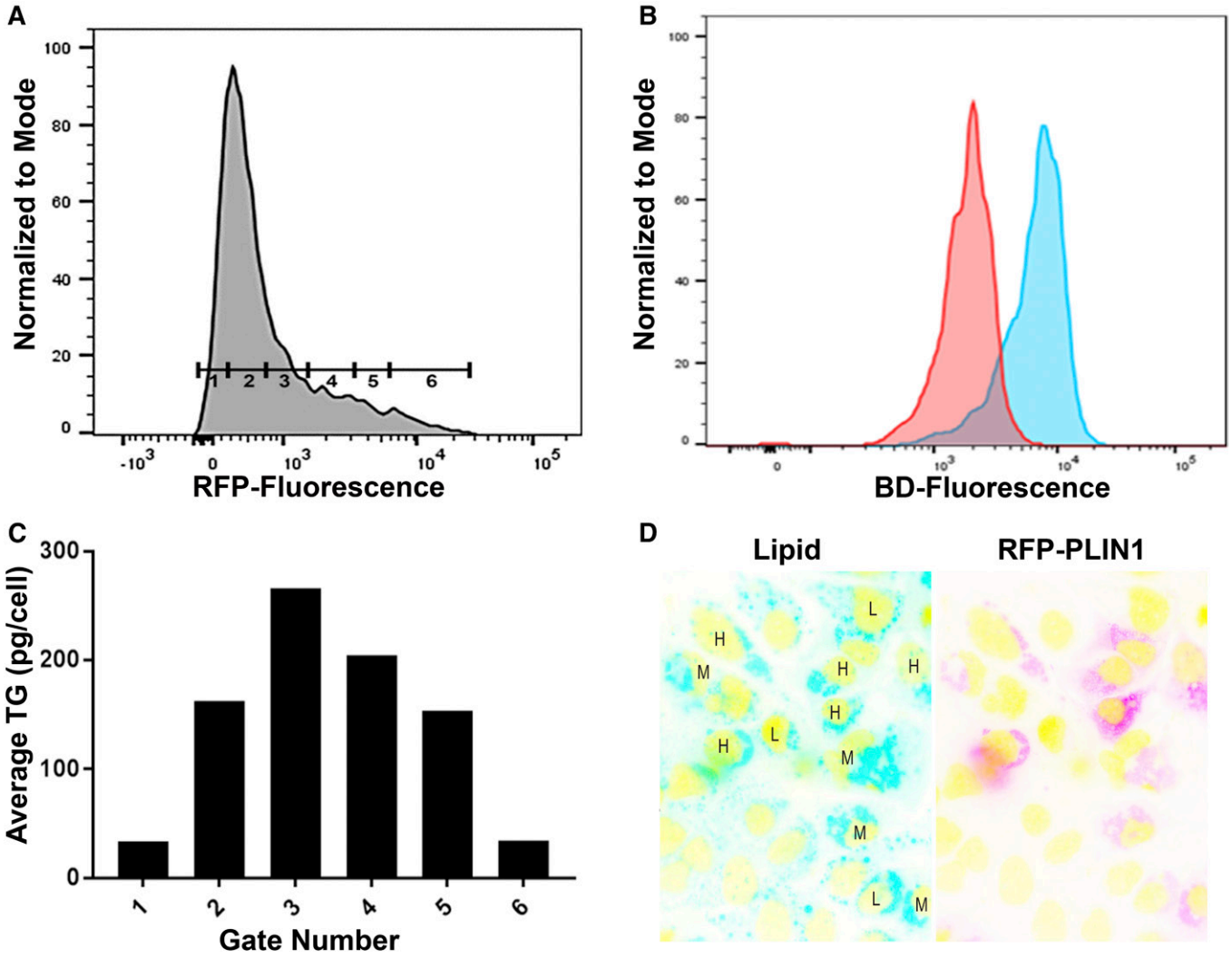

Fig. 5. RFP-PLIN1 transfected Huh7 cells store more TG. Huh7 cells were transfected with RFP-PLIN1 as described in the Materials and Methods. Cells were then incubated with 100 μM FA (complexed 5.5:1 with BSA) for 18 h and fixed. A: Histogram of RFP signal with six gates. B: Histogram of BD fluorescence from panel A gates 1 (red) and 3 (blue). C: Estimate of TG mass of cells from the six gates shown in panel A. The Huh7 cells with known TG mass (shown in Fig. 3A) were assessed in the same FC session as the RFP-PLIN1-transfected Huh7 cells. TG mass versus BDMFI was plotted and the best fit line was determined. This line was used to estimate TG mass in the RFP-PLIN1 transfected Huh7 cells. D: Huh7 cells transfected with RFP-PLIN1 were treated in parallel as those used for FC. The lipid panel shows the BD channel and the DAPI channel and PLIN1 shows the RFP and the DAPI channel. The BD signal is teal, RFP signal is pink, and DAPI signal is yellow. H, M, and L indicate high, medium and low levels of RFP.

levels and these other signals can be assessed in a single cell-type in a mixed cell population. This context allowed the novel observation that the previously described PLIN1-driven TG accumulation requires only low levels of PLIN1 and higher PLIN1 levels have a lesser effect on TG accumulation than lower levels (Fig. 5).

BD is hydrophobic and labels lipid by partitioning into the cellular lipid. This partitioning is independent of biologic processes. Because BD partitions into all hydrophobic compartments, it detects total lipid mass with little regard to neutral lipid types. Because TG is the long-term energy store for animals, it represents the bulk of the lipid in most animal tissues. Thus, TG and total lipid mass usually are similar. However, this is not true for some specialized cells that have a significant fraction of other neutral lipid species, such as monoalkyl-diacylglycerol, cholesterol esters, and retinal esters. This method allows total lipid to be measured in a single cell type, which may be dissimilar to neutral lipid composition of the tissue. For example, adipose macrophage neutral lipid composition may have little resemblance to the whole adipose tissue. However, because a detailed understanding of the lipid composition within a cell is resource intensive and total lipid levels (steatosis) often correlate with metabolic disease (1–5), this single parameter description of lipid levels may be advantageous.

This method allows estimation of lipid mass by using cells of similar size and known BDMFI and average TG mass/cell. Figure 3A shows that cell size affects non-TG-dependent BDMFI (the intercept of the line), but does not affect the TG-dependent increase in BDMFI. This can allow reasonable estimates of cellular TG mass. However, it is important to have similar nonlipid BDMFI, especially in lean cells where the ratio of nonlipid to lipid BDMFI is high. For example, estimating lipid mass in the

human monocytes shown in Fig. 4 illustrates this concept. The larger cultured monocytes have a higher nonlipid BDMFI than primary monocytes and, as a result, too little of the BDMFI in primary monocytes is attributed to lipid, making the interpolated lipid mass an underestimate. Using this strategy appropriately, we show that RFP-PLIN1 expression increased TG accumulation, which was previously observed (12). However, this experiment also indicates that accumulation is maximal at moderate RFP-PLIN1 levels and very high levels of expression inhibit TG accumulation (Fig. 5C). Despite the intensive study of PLIN1, this has not been reported, although, as mentioned above, in vivo studies showed that overexpression diminished adipocyte TG stores (14). This strategy makes it feasible to assess the effect on lipid levels of proteins that cannot be stably expressed. In addition, the relative potency of different proteins on lipid levels can be assessed by using the same fluorescent tag (e.g., RFP) on each protein to determine and match the levels of the proteins of interest.

In summary, we report a method to assess lipid mass in single cells by FC. The utility of this method is enhanced by existing FC methods and reagents to assess many cellular parameters (e.g., apoptosis, cholesterol levels, and surface antigens) that can be contemporaneously employed. Further, other neutral lipid staining fluorophores with different spectrums can be utilized when autofluorescence or the measurements of other parameters preclude the use of the FITC channel.

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REFERENCES

1. Unger, R. H. 2003. Lipid overload and overflow: metabolic trauma and the metabolic syndrome. *Trends Endocrinol. Metab.* **14:** 398–403.
2. Unger, R. H., G. O. Clark, P. E. Scherer, and L. Orci. 2010. Lipid homeostasis, lipotoxicity and the metabolic syndrome. *Biochim. Biophys. Acta.* **1801:** 209–214.
3. Unger, R. H., and P. E. Scherer. 2010. Gluttony, sloth and the metabolic syndrome: a roadmap to lipotoxicity. *Trends Endocrinol. Metab.* **21:** 345–352.
4. Fabbrini, E., F. Magkos, B. S. Mohammed, T. Pietka, N. A. Abumrad, B. W. Patterson, A. Okunade, and S. Klein. 2009. Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. *Proc. Natl. Acad. Sci. USA.* **106:** 15430–15435.
5. Fabbrini, E., S. Sullivan, and S. Klein. 2010. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic and clinical implications. *Hepatology.* **51:** 679–689.
6. Steinbring, J., A. Graja, A-M. Jank, and T. J. Schulz. 2017. Flow cytometric isolation and differentiation of adipogenic progenitor cells into brown and brite/beige adipocytes. *In* Thermogenic Fat: Methods and Protocols. Vol. 1566. J. Wu, editor. Humana Press, New York. 25–36.
7. Listenberger, L. L., A. M. Studer, D. A. Brown, and N. E. Wolins. 2016. Fluorescent detection of lipid droplets and associated proteins. *Curr. Protoc. Cell Biol.* **71:** 4.31.1–4.31.14.
8. Wolins, N. E., J. R. Skinner, M. J. Schoenfish, A. Tzekov, K. G. Bensch, and P. E. Bickel. 2003. Adipocyte protein S3-12 coats nascent lipid droplets. *J. Biol. Chem.* **278:** 37713–37721.
9. Schwartz, D. M., and N. E. Wolins. 2007. A simple and rapid method to assay triacylglycerol in cells and tissues. *J. Lipid Res.* **48:** 2514–2520.
10. Campbell, R. E., O. Tour, A. E. Palmer, P. A. Steinbach, G. S. Baird, D. A. Zacharias, and R. Y. Tsien. 2002. A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA.* **99:** 7877–7882.
11. Miura, S., J. W. Gan, J. Brzostowski, M. J. Parisi, C. J. Schultz, C. Londos, B. Oliver, and A. R. Kimmel. 2002. Functional conservation for lipid storage droplet association among perilipin, ADRP, and TIP47 (PAT)-related proteins in mammals, Drosophila, and Dictyostelium. *J. Biol. Chem.* **277:** 32253–32257.
12. Brasaemle, D. L., B. Rubin, I. A. Harten, J. Gruia-Gray, A. R. Kimmel, and C. Londos. 2000. Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. *J. Biol. Chem.* **275:** 38486–38493.
13. Martinez-Botas, J., J. B. Anderson, D. Tessier, A. Lapillonne, B. H-J. Chang, M. J. Quast, D. Gorenstein, K-H. Chen, and L. Chan. 2000. Absence of perilipin results in leanness and reverses obesity in Leprdb/db mice. *Nat. Genet.* **26:** 474–479.
14. Miyoshi, H., S. C. Souza, M. Endo, T. Sawada, J. W. Perfield, C. Shimizu, Z. Stancheva, S. Nagai, K. J. Strissel, N. Yoshioka, et al. 2010. Perilipin overexpression in mice protects against diet-induced obesity. *J. Lipid Res.* **51:** 975–982.
15. Tansey, J. T., C. Sztalryd, J. Gruia-Gray, D. L. Roush, J. V. Zee, O. Gavrilova, M. L. Reitman, C. X. Deng, C. Li, A. R. Kimmel, et al. 2001. Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. *Proc. Natl. Acad. Sci. USA.* **98:** 6494–6499.