Real-time quantification of fatty acid uptake using a novel fluorescence assay

Jinfang Liao,* Richard Sportsman,* Jeff Harris,* and Andreas Stahl[1,†,§]

Molecular Devices Corporation,* Sunnyvale, CA 94089; Palo Alto Medical Foundation Research Institute,† Palo Alto, CA 94301; and Division of Gastroenterology/Hepatology,§ Stanford University School of Medicine, Stanford, CA 94305

Abstract Uptake of nonesterified long-chain fatty acids (LCFAs) into many cell types and organs such as liver, heart, intestine, and skeletal muscle occurs primarily through a saturable, protein-mediated mechanism. Membrane proteins that increase the uptake of LCFAs, such as FAT/CD36 and fatty acid transport proteins, represent significant therapeutic targets for the treatment of metabolic disorders, including type 2 diabetes. However, currently available methods for the quantification of LCFA uptake neither allow for real-time measurements of uptake kinetics nor are ideally suited for the development of LCFA uptake inhibitors in high-throughput screens. To address both problems, we developed a LCFA uptake assay using a fluorescently labeled fatty acid and a nontoxic cell-impermeable quenching agent that allows fatty acid transport to be measured in real time using fluorescence plate readers or standard fluorescence microscopy. With this assay, we faithfully reproduced known differentiation- and hormone-induced changes in LCFA uptake by 3T3-L1 cells and determined LCFA uptake kinetics with previously unobtainable temporal resolution. Applications of this novel assay should facilitate new insights into the biology of fatty acid uptake and provide new means for obesity-related drug discovery.—Liao, J., R. Sportsman, J. Harris, and A. Stahl. **Real-time quantification of fatty acid uptake using a novel fluorescence assay. *J. Lipid Res.* 2005.** 46: **597–602.**

Supplementary key words long-chain fatty acids • fatty acid uptake assay • quencher dyes

Uptake of long-chain fatty acids (LCFAs) plays an important role in the absorption of dietary lipids as well as the delivery of metabolic energy to a variety of tissues. Besides their function as substrates for β-oxidation, fatty acids also contribute to membrane synthesis, protein modifications, immune responses, and activation of protein kinases and nuclear hormone receptors (1, 2). Recent findings have also directly implicated increased intracellular levels of LCFAs in the obesity-associated insulin desensitization of skeletal muscle and liver (1, 3).

LCFA uptake in adipocytes is mainly facilitated by membrane proteins, particularly members of the fatty acid transport protein (FATPs/SLC27) family. Mammalian genomes have been shown to contain six FATP genes (4). The identification of this fatty acid transporter family and other fatty acid uptake-enhancing proteins such as CD36 has allowed a better understanding of the mechanisms and regulation of LCFA transport on a cellular level, yielding insight into the control of energy homeostasis and its dysregulation in diseases such as diabetes and obesity. In addition, these cell surface proteins represent new targets for the inhibition of LCFA uptake.

To gain a better understanding of the molecular dynamics of fatty acid uptake and the development of small molecular inhibitors of this process, robust and physiologically relevant measurements of LCFA uptake kinetics are required. Here, we report the development of a fluorescence assay based on the extracellular quenching of a fluorescent fatty acid analog that can be quantified in real time by fluorescence plate readers or by standard fluorescence microscopy-based systems without a washing step. Using this assay, we measured differentiation- and hormone-induced changes in LCFA uptake by 3T3-L1 cells in a manner consistent with other methods. Using the same assay in combination with an automated microscopy system, we were able to both visualize and quantify cellular fatty acid uptake and accumulation in different subcellular locations. This new assay offers both the benefit of detailed real-time observations and measurements of cellular fatty acid uptake and a simple mix-and-read format for high-throughput screens.

MATERIALS AND METHODS

Reagents

All reagents were from Sigma with the exception of the Quencher-Based Technology (QBT) Fatty Acid Uptake Assay Kit (Molecular Devices).

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[1] To whom correspondence should be addressed.
e-mail: astahl@stanford.edu

QBT Fatty Acid Uptake reagent

The QBT Fatty Acid Uptake Assay Kit consisted of a proprietary formulation of the quenching agent Q-Red.1 (Molecular Devices) and 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*S*-indacene-3-dodecanoic acid (BODIPY-FA; Molecular Probes, Inc.). QBT Fatty Acid Uptake Assay stock solutions were dissolved completely by adding 10 ml of 1× HBSS buffer (1× Hank's balanced salt solution with 20 mM HEPES and 0.2% fatty acid-free BSA).

Cell culture and treatment

3T3-L1 fibroblasts (ATCC) were grown in DMEM containing 10% fetal bovine serum with 2 mM L-glutamine and 1% penicillin/streptomycin (DMEM/FBS). Differentiated cells were prepared as described previously (5, 6). In short, 3T3-L1 fibroblasts were grown 2 days after confluence in DMEM/FBS and then for 2 days in DMEM/FBS supplemented with 0.83 μM insulin, 0.25 μM dexamethasone, and 0.25 mM isobutylmethylxanthine. The medium was then changed to DMEM/FBS supplemented with 0.83 μM insulin for 2 days only and then maintained in DMEM/FBS alone for a further 3–5 days. Differentiated cells (at least 95% of which showed an adipocyte phenotype by accumulation of lipid droplets) were used on days 8–12 after initiation of differentiation.

Fluorescence-activated cell sorter (FACS)-based fatty acid uptake assays

3T3-L1 fibroblasts or adipocytes were seeded onto six-well plates. Cells were preincubated with Q-Red.1 for 15 min. BODIPY-FA (2 μM in HBSS with 0.1% BSA) was added to each well for 1 min at 37°C. Cells were placed on ice and washed with cold-stop solution and detached with trypsin/EDTA. Cells were resuspended in cold fatty acyl-CoA (FACS) buffer with propidium iodine (PI) and analyzed on a FACScalibur (Becton, Dickinson & Co.) by determining FL-1 fluorescence of PI-negative cells. Viability was determined from the ratio of PI-negative to PI-positive cells.

Fatty acid uptake on microplates

3T3-L1 adipocytes and fibroblasts were plated onto a 96-well black-wall/clear-bottom plate (Costar) at 50,000–80,000 cells/well in 100 μl of DMEM/FBS for 4–5 h. Cells were then serum deprived for 1 h before treatment with insulin for 30 min (see figure legends) followed by the addition of QBT Fatty Acid Uptake solution to the well (experiments with the medium replaced with HBSS were compared). For inhibition and competition experiments, fatty acids and other compounds were directly added to the QBT Fatty Acid Uptake mix. Kinetic readings were started immediately with a Gemini or Flexstation fluorescence plate reader (Molecular Devices) at 37°C. Instrument settings were as follows: bottom read, medium sensitivity, excitation 488/emission 515, with a filter cutoff at 495 nm. Integrated intensity readings at a given time point, calculated by Softmax Pro® software (Molecular Devices), represent essentially the sum (i.e., the area under the curve) up to that point.

Microscopic imaging of fatty acid translocation

To visualize the effect of Q-Red.1 addition to BODIPY-FA uptake solutions, 3T3-L1 adipocytes prepared as above on 96-well black-wall/clear-bottom plates were loaded with BODIPY-FA for 5 min in HBSS buffer followed by the addition of Q-Red.1 (Molecular Devices). A series of images were taken on a Discovery-1 imaging microscope (Molecular Devices) before and after adding the quencher reagent. To monitor real-time fatty acid uptake and accumulation, MetaMorph software was used to take continuous recordings of cells immediately after replacing the medium with 100 μl of QBT Fatty Acid Uptake reagent.

RESULTS

Q-Red.1 quenches BODIPY-FA fluorescence without affecting cell viability or LCFA uptake of 3T3-L1 adipocytes

To determine the effective concentration of Q-Red.1, we titrated the compound against a 2 μM BODIPY-FA solution and measured fluorescence using a Gemini plate reader. **Figure 1A** shows that at a 100 μM concentration of Q-Red.1, more than 99% of the fluorescence in the solution was quenched. To exclude the possibility that Q-Red.1 either di-

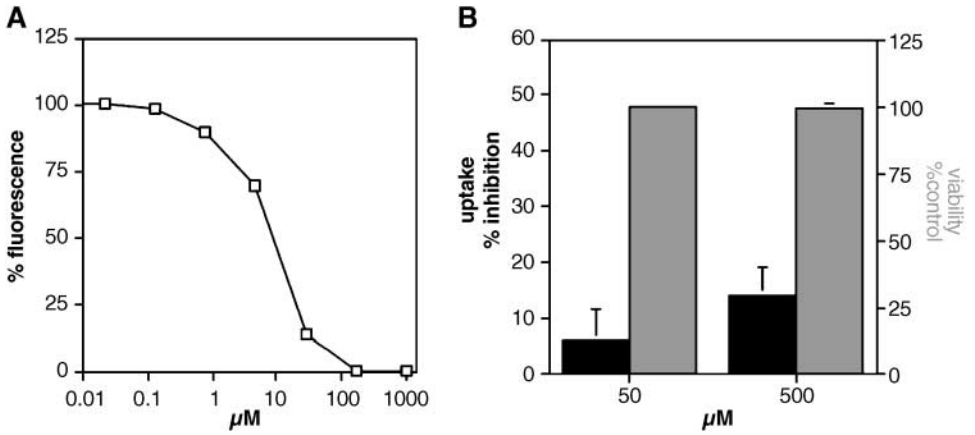

Fig. 1. Effect of Q-Red.1 on 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*S*-indacene-3-dodecanoic acid (BODIPY-FA) fluorescence, uptake, and cell viability. A: Fluorescence at 510 nm of a 2 μM aqueous solution of BODIPY-FA in the presence of the indicated amounts of Q-Red.1. Zero percent and 100% were defined as fluorescence of 0 or 2 μM BODIPY-FA, respectively. Measurements were done in quadruplicate. B: Flow cytometry-based determination of alterations in long-chain fatty acid (LCFA) uptake (black bars, left axis) and cell viability (gray bars, right axis) caused by the indicated concentration of Q-Red.1. Zero percent inhibition and 100% viability was based on control samples without Q-Red.1. Measurements were done in triplicate. Error bars represent average of triplicate measurements.

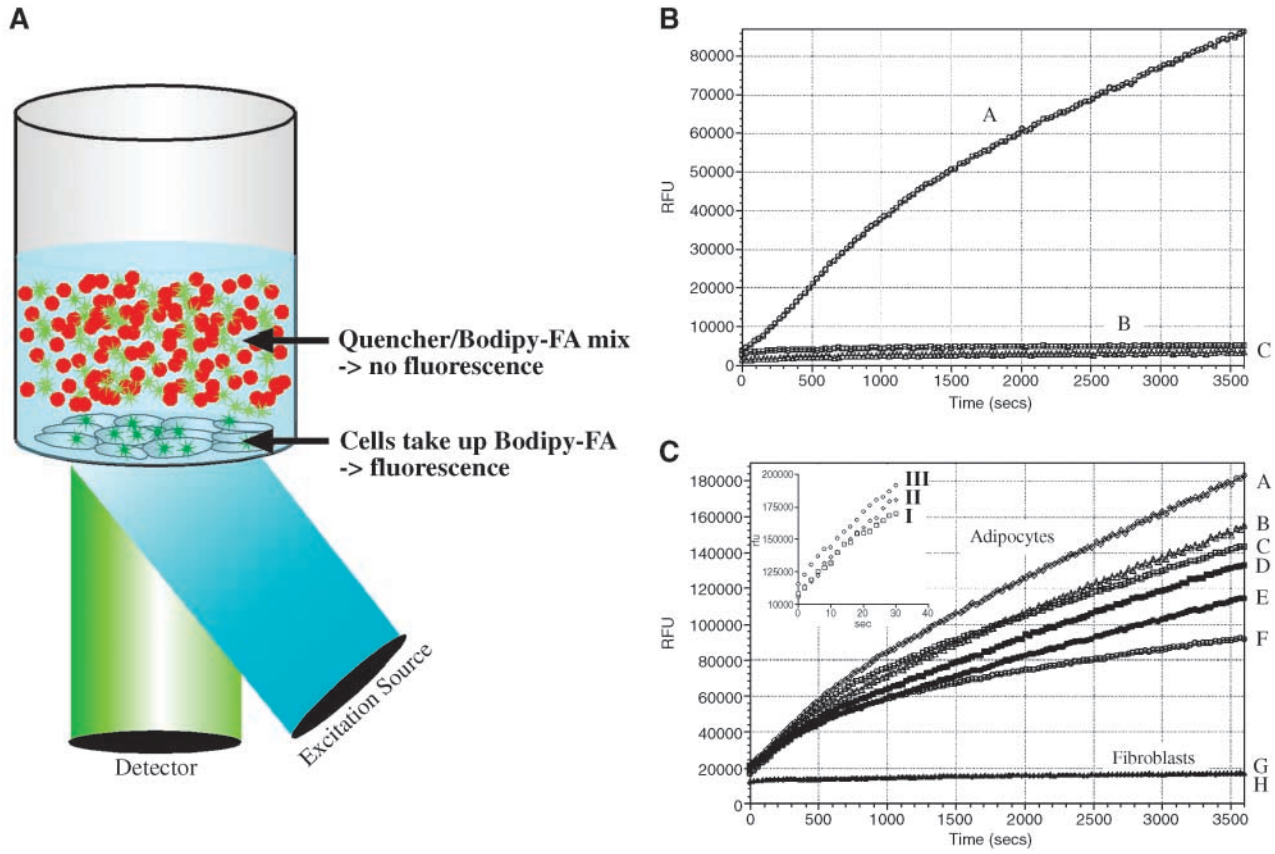

Fig. 2. Effect of differentiation and insulin on fatty acid uptake by 3T3-L1 cells. A: Schematic of the Quencher-Based Technology (QBT) Fatty Acid Uptake Assay. BODIPY-FA fluorescence is quenched in solution by Q-Red.1. Adiopocytes actively take up the fluorescent fatty acids but not the quencher. Subsequently, intracellular BODIPY-FA fluorescence can be measured using a detector positioned beneath the plate. B: Comparison of kinetic readings with a Flexstation plate reader of LCFA uptake by adipocytes (A), fibroblasts (B), and no cells (C; uptake mixture only). C: Insulin-induced fatty acid uptake by 3T3-L1 adipocytes and fibroblasts was assessed by incubation of serum-starved cells for 30 min with varying concentrations of insulin. At the end of the incubation time, 100 μl of QBT Fatty Acid Uptake reagent was added to each well, and kinetic readings were started immediately with a Flexstation plate reader. Traces A to F correspond to adipocytes with 160, 16, 8, 1.6, 0.16, and 0 nM insulin, respectively; traces G and H correspond to fibroblasts with 160 and 0 nM insulin, respectively. The inset shows the first 30 s of uptake with 0 nM (I), 1 nM (II), and 100 nM (III) insulin. RFU, Relative Fluorescence Unit.

rectly interferes with cellular LCFA uptake or has unspecific toxic effects on cells, we incubated 3T3-L1 adipocytes for 30 min with the indicated concentrations of the quencher (Fig. 1B) and subsequently determined the uptake of BODPY-FA and viability via FACS after removal of the quencher. At the highest concentration used (500 μM), the quencher interfered with FACS-based fatty acid uptake detection only minimally and had no impact on viability (Fig. 1B).

QBT-based assays can detect differentiation and insulin-induced changes of fatty acid uptake in real time

Fatty acid uptake is significantly induced during 3T3-L1 differentiation from fibroblasts to adipocytes (7). To reproduce this known change in fatty acid uptake velocity, we seeded fully differentiated 3T3-L1 adipocytes and undifferentiated fibroblasts onto 96-well plates and recorded changes in fluorescence after addition of the QBT Fatty Acid Uptake reagent on a Flexstation plate reader. In this setting, the assay is based on the bottom excitation and emission detection of BODIPY fluorescence at 488 and 515 nm, respectively. The oblique excitation source fully illuminates the cells at the bottom of the well and some of the quencher/BODIPY-FA mix (200 and 2 μM, respectively) above the cells (**Fig. 2A**). Therefore, some of the BODIPY-FA is excited but the quencher effectively suppresses its fluorescence. Adipocytes robustly take up BODIPY-FA, likely through protein-mediated transport, but exclude the quench reagent. Inside the cells, therefore, BODIPY-FA becomes unquenched and its fluorescence can be quantified by a bottom detector (Fig. 2A).

Although 3T3-L1 fibroblasts showed a slow linear increase of fluorescence over time (integrated intensity of 1.64e7 (1.64×10^{-7}) relative fluroescence unit (RFU) for 60 min), LCFA uptake by adipocytes was initially rapid ($1.1e^6$ RFU/min for first 10 min) and showed nonlinear kinetics at later time points (integrated intensity of $1.88e^8$ RFU over 60 min) (Fig. 2B). To further compare our new LCFA uptake assay with known effectors of fatty acid uptake, we preincubated 3T3-L1 adipocytes or fibroblasts with varying concentrations of insulin for 30 min. Figure 2C shows that fluorescence intensity increased with in-

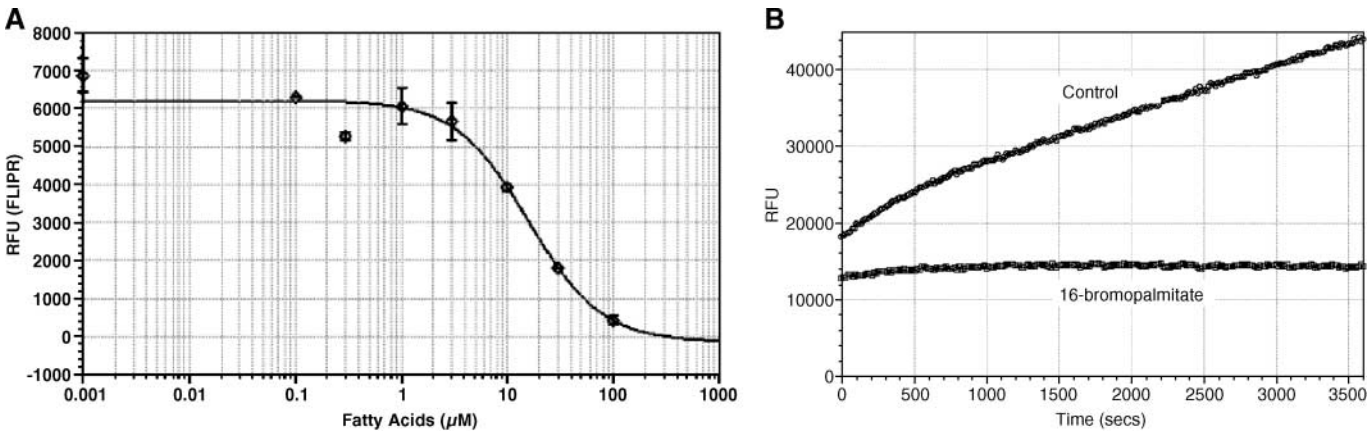

Fig. 3. Inhibition of BODIPY-FA uptake by natural and synthetic fatty acids. A: Integrated intensity readings after a 10 min kinetics run using 3T3-L1 adipocytes and a FLIPR station. QBT Fatty Acid Uptake reagent contained 0.2% BSA and 0, 0.1, 0.3, 1, 3, 10, 30, or 100 μM palmitate. B: LCFA uptake measured with QBT Fatty Acid Uptake without (control) or with 125 μM 16-bromopalmitate and 0.2% BSA. FLIPR, Fluorometric Imaging Plate Reader; RFU, Relative Fluorescence Unit.

creased dose of insulin in adipocytes, but not fibroblasts, in accordance with previous reports (6). Two second interval readings during the initial linear phase of uptake also revealed increased fatty acid uptake by insulin-stimulated adipocytes (Fig. 2C, inset). From the end point readings at 60 min using the data in Fig. 2C, we determined an EC_{50} for insulin-stimulated uptake of BODIPY-FA of 2 nM. In an independent series of experiments, insulin was added at the beginning of the assay without preincubation. Uptake curves for insulin-treated uptake diverged significantly from untreated cells after 4 min (data not shown). These experiments demonstrate that QBT Fatty Acid Uptake Assays in combination with fluorescence plate readers allow for sensitive real-time detection of modulations in LCFA uptake in a high-throughput format.

BODIPY-FA uptake can be competed for by natural fatty acids and inhibited by synthetic compounds

BODIPY-FA uptake closely correlates with the uptake of radiolabeled fatty acids (6, 8) and is incorporated into both neutral lipids and phospholipids (9), suggesting that at least the initial steps of uptake and processing are shared between fluorescently labeled and unlabeled LCFAs. To further confirm this notion, we wanted to test whether the novel uptake assay could be used to determine the ability of unlabeled fatty acids to compete for the uptake of BODIPY-FA. Indeed, addition of palmitate to the quencher mix inhibited BODIPY-FA uptake in a dose-dependent manner with an apparent IC_{50} of 15.8 μM (**Fig. 3**). Similarly, we were able to use the QBT Fatty Acid Uptake Assay to determine LCFA uptake inhibition by synthetic compounds such as 16-bromopalmitate (Fig. 3B) and 2-bromopalmitate (data not shown). These results are consistent with the hypothesis that BODIPY-FA uptake is mediated by the same mechanisms that facilitate the uptake of naturally occurring fatty acids and therefore can be used to detect competitive or irreversible inhibition of cellular LCFA uptake by synthetic compounds.

The QBT Fatty Acid Uptake Assay can be used for real-time imaging of cellular fatty acid uptake

The high fluorescence of micromolar solutions of BODIPY-FA prevents imaging of the rapid cellular uptake process into adipocytes (**Fig. 4A**, first image). Addition of Q-Red.1 to a final concentration of 100 μM efficiently and rapidly quenched the fluorescence of BODIPY-FA in solution, revealing the intracellular accumulation of fatty acids in adipocytes (Fig. 4A). To further demonstrate the utility of the QBT assay for fluorescent light imaging of LCFA uptake, we recorded the initial 10 min of BODIPY-FA uptake by several 3T3-L1 adipocytes (Fig. 4B). The recorded data could be further analyzed by specifying different regions of interest, such as lipid droplets of different cells (Fig. 4C, left; blue, yellow, green), cytoplasm (magenta), and cell-free background (turquoise). Subsequent evaluation of fluorescence intensity over unit area for the specified regions (Fig. 4B, right) can then be used to quantitatively follow the intracellular fate of fatty acids.

DISCUSSION

Current methods for the determination of fatty acid uptake include end point assays with radiolabeled fatty acids (10), *cis*-parinaric acid, BODIPY- and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino (NBD)-conjugated fatty acids (11), intracellular pH measurements (12), and measurements of LCFA binding to microinjected fluorescent fatty acid binding proteins (13). None of these techniques allow for the continuous measurement of LCFA uptake into cells, nor are they practical for high-throughput screens. Although chemically identical to naturally occurring fatty acids, radiolabeled compounds are expensive, not compatible with high-throughput screens, and can only be used in end point assays. *Cis*-parinaric acid is a naturally occurring polyunsaturated fatty acid that fluoresces upon protein binding. Therefore, it is principally suitable for

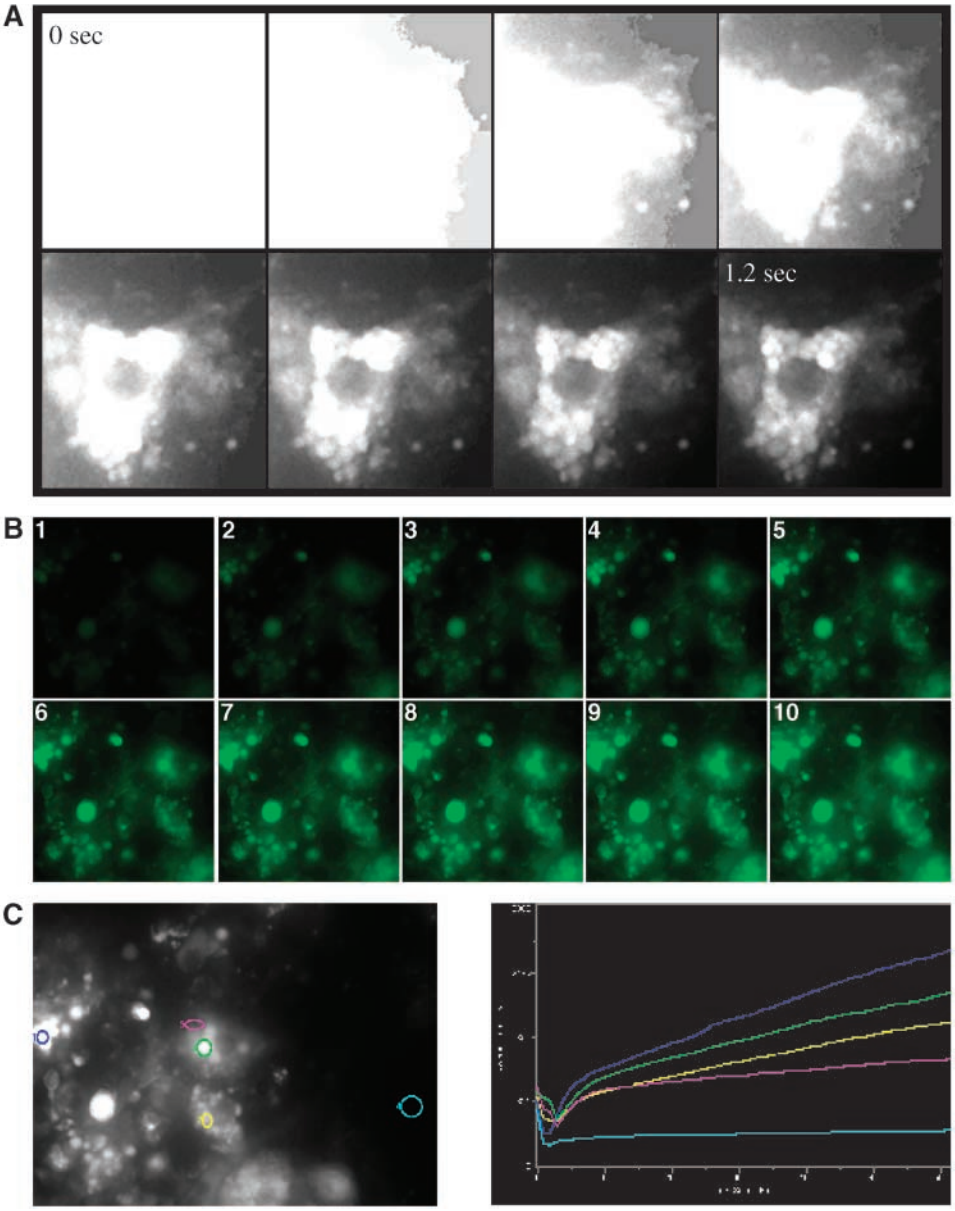

Fig. 4. Use of Q-Red.1 in fluorescence microscopy. A: Image series showing the first 1.2 s after addition of Q-Red.1 to adipocytes incubated with 2 μM BODIPY-FA. B: Image series taken at 1 min intervals after addition of the QBT Fatty Acid Uptake reagent to 3T3-L1 adipocytes. C: Quantification of BODIPY-FA uptake depicted in B. Areas for measurements are shown at left as lipid droplets in three different cells (blue, yellow, and green), cell-free background (turquoise), and cytoplasm (magenta). Changes in fluorescence intensity/area were then plotted over the 10 min time course (right).

real-time uptake assays; however, binding to extracellular or intracellular proteins cannot be distinguished by this method, and BSA, the physiological carrier of LCFAs in the serum, cannot be used. BODIPY and NBD fatty acid conjugates have been widely used as partially metabolizable fatty acid analogous. However, their use has been limited to end point assays, which require cells to be washed to remove excess compound, often resulting in the detachment of cells on high-density plates. Lastly, measurements of intracellular pH changes attributable to fatty acid translocation are a very indirect method that is not necessarily coupled to fatty acid uptake.

To address these problems, we developed a novel LCFA uptake assay, the QBT Fatty Acid Uptake Assay, which combines the fluorescently labeled fatty acid BODIPY-FA with the novel quenching agent Q-Red.1. This kit is a nonwash assay that allows LCFA transport to be measured in real time on 96- or 384-well microplate formats.

We have developed and tested a number of highly purified quenching agents that are nontoxic and nonpermeable to live cells. In preliminary experiments, we found that among these Q-Red.1 is particularly useful in this assay because its absorption spectrum overlaps well with the emission wavelengths of the BODIPY-FA fluorescently labeled fatty acid. These qualities set Q-Red.1 apart from previously used quenchers such as trypan blue and crystal

violet (14), which demonstrate inferior quenching capabilities, often permeate cells, show significant cytotoxicity at higher concentrations, and can be chemically ill-defined, causing significant differences between batches and manufacturers (15, 16). Minor interference of high concentrations (500 μM) of Q-Red.1 with BODIPY-FA uptake by adipocytes as assessed by FACS was likely attributable to internalization of the quencher, possibly by pinocytosis, because distinct red vesicles could be observed within the cells.

Using the novel QBT Fatty Acid Uptake Assay, we obtained data for differentiation- and insulin-induced changes in LCFA uptake by 3T3-L1 cells that are consistent with previous results derived using standard techniques (6, 7). Also, real-time uptake kinetics for BODIPY-FA reported here closely resembled measurements of LCFA uptake into adipocytes monitored by changes in intracellular fatty acid levels (13). Together with our finding that palmitate can quantitatively compete with the uptake of BODIPY-FA and the fact that BODIPY-FA becomes incorporated into cellular lipids (9), this strongly supports the notion that BODIPY-FA uptake truthfully mimics the uptake of naturally occurring LCFAs.

Additionally, we demonstrated that the same quenching technology can be expanded to the observation of cellular LCFA uptake by fluorescence microscopy. As our preliminary results show, the method can be easily used to determine the kinetics of intracellular fatty acid shuttling and will provide a valuable tool to further dissect the fate of intracellular fatty acids after uptake. In summary, we have developed a fast, simple, and reliable fluorescence-based assay for the detection of fatty acid uptake in adherent cells that can be expanded to observe this process by fluorescence microscopy. Adaptation of the QBT Fatty Acid Uptake assay to screen for inhibitors of fatty acid transport should provide a useful drug discovery tool and may lead to new insight into the kinetics and cell biology of fatty acid uptake into a variety of cell types.

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