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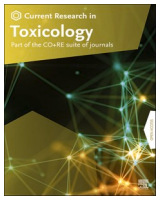

A simple, reliable and easily generalizable cell-based assay for screening potential drugs that inhibit lipid accumulation

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ABSTRACT

Ectopic lipid deposition in the hepatocyte plays an important role in the development of nonalcoholic fatty liver disease (NAFLD), which has become one of the most common causes of chronic liver disease worldwide yet no approved drugs are currently available. In this study, a cell-based method was developed to screen potential drugs with low toxicity that inhibit lipid accumulation. In the same 96-well plate, cytotoxicity was measured using CCK8 assay, followed by lipid content detection using BODIPY 493/503 via fluorometry assay, a lipid droplet-specific fluorescent dye commonly used in microscopy and flow cytometry, but not previously reported in fluorometry. Lipid content was normalized to DAPI staining to control for cell number. The results of this assay were highly consistent with the fluorescence microscopy, with significantly lower intra-group variability in detecting lipid accumulation induced by free fatty acids in Huh7 cells. Validation was conducted using 10 well documented steatotic compounds and 5 negative controls, all of which were correctly identified by the assay. In addition, the inhibitory effect of ML261, a well-known inhibitor of hepatic lipid droplets formation, was also confirmed by the assay both in AML12 cells and Hepa1-6 cells. To our knowledge, this study is the first to quantify lipid droplets using BODIPY 493/503 by fluorometry assay, and to demonstrate that CCK8 does not interfere with subsequent BODIPY 493/503 staining, both of which will reduce the cost and increase the efficiency. In conclusion, the method is simple, reliable, efficient and does not rely on expensive instruments, making it an easily generalizable approach to identify potential drug candidates for NAFLD treatment.

Introduction

Nonalcoholic fatty liver disease (NAFLD), defined as steatosis where intrahepatic lipid content exceeds 5 % of liver wet weight without substantial alcohol consumption, viral hepatitis or other causes of chronic liver disease (CLD), has become one of the most common causes of CLD worldwide (Yki-Järvinen et al., 2021; Quek et al., 2023). According to the latest literature, NAFLD affects up to about 38 % of the adult population worldwide, and its prevalence is increasing in parallel with the global obesity epidemic, resulting an estimated about 50 % the worldwide adult population is forecasted to have NAFLD by 2040. More and more clinical evidence demonstrated that NAFLD may increase the incidence of a variety of extrahepatic complications like type 2 diabetes mellitus (T2DM), hypertension (HTN) and cardiovascular disease (CVD), resulting a significant social and economic burden (Huang et al., 2023; Israelsen et al., 2024; Liu et al., 2024; Mantovani et al., 2024; Souza et al., 2024). What's worse, NAFLD is a multifactorial condition, which driven by multiple factors such as overweight, T2DM, obese, dyslipidemia or some steatosis-induced drugs (Israelsen et al., 2024; Younossi and Stepanova, 2024), with increasing prevalence worldwide and a high unmet medical need, as no specific surgical interventions or approved drugs are available for its treatment (Harrison et al., 2023; Verrastro et al., 2023). Additionally, many currently used drugs are known to induce steatosis, which could disrupt clinical trials or the result in market withdrawal (Müller et al., 2023).

Several *in vitro* assays have been developed to assess drug-induced steatosis in cultured primary hepatocytes or hepatoma cell lines by detecting intracellular lipids content, identifying early proteomic markers, or analyzing the expression profiles of lipid metabolism-related gene and transcription factors (Meneses-Lorente et al., 2006; Donato et al., 2009; Fujimura et al., 2009; Donato et al., 2012; Tolosa et al., 2016; Alarcan et al., 2021; Müller et al., 2023). As fatty liver is a complex disease with multiple pathogenic factors, directly quantifying lipid droplets provides a more reliable approach to screen drugs for steatosis.

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Nowadays, a few methods based on lipid quantification, such as using Oil Red O, Nile Red or BODIPY 493/503, are combined with flow cytometry or automatic cell image analysis (Meneses-Lorente et al., 2006; Donato et al., 2009; Fujimura et al., 2009; Donato and Gómez-Lechón, 2012; Donato et al., 2012). However, these methods are either cumbersome to operate or rely on expensive instruments, limiting their widespread use. There are also a few studies using Nile Red (Sugarman et al., 2014; Lee et al., 2022), AdipoRed (Alarcan et al., 2021) or BODIPY558/568 C_{12} (Fujimura et al., 2009) combined with fluorometry, but each has its limitations. For example, Nile Red stains both neutral lipids and phospholipids, reducing its specificity. BODIPY 493/503 is a lipid droplet-specific fluorescent dye widely used in fluorescence microscopy and flow cytometry, but its suitability for fluorometry has not yet been established.

Among the reported methods for evaluating drug-induced steatosis, propidium iodide (PI) (Donato et al., 2012), water-soluble tetrazolium 1 (WST-1) assay (Müller et al., 2023), methyl-thiazolyl-tetrazolium (MTT) (Meneses-Lorente et al., 2006; Donato et al., 2009; Lee et al., 2022), or lactate dehydrogenase (LDH) assay (Fujimura et al., 2009) were used to assess cell viability for drug toxicity. However, cell counting kit-8 (CCK8) assay has not been reported. PI requires cell fixation, and MTT requires dissolution in organic solvents, both of which precent the maintaining of cell activity. Although LDH assay preserves cell activity, it requires supernatant collection, making it time-consuming and laborious. Both WST-1 and CCK-8, in contrast, have no significant cytotoxicity and do not require additional steps like supernatant collection, cell washing, cell collection, or solvent dissolve, giving them clear advantages over PI, MTT, and LDH assay. CCK-8, in particular, is more stable, sensitive, soluble, and easier to preserve than WST-1, making it an excellent candidate for cell viability (Lutter et al., 2017).

In the current study, we aim to develop a simple and reliable 96-well cell-based assay to screen drugs or new compounds that inhibit lipid deposition, without relying on expensive equipment, making it easy to implement. Firstly, to validate the utility of BODIPY493/503 in determining lipid droplets by fluorometry, the human hepatoma cell line Huh7 cells were treated with free fatty acids (FFAs) or an equal amount of solvent. The lipid droplets were quantified by fluorometry and fluorescence microscopy assay respectively with BODIPY 493/503, and the relative quantitative results between groups and the intra-group variability of the two methods were compared. Secondly, Huh7 cells were treated with FFAs or an equal amount of solvent, and it was evaluated whether CCK8 interfered with the subsequent relative quantitation of lipid droplets by BODIPY 493/503. Finally, we used 10 well-known steatosis inducers, 5 negative controls, and ML261, a well-known hepatic lipid droplets formation inhibitor, to verify the reliability of this newly developed method.

Materials and methods

Cell culture and reagents

The human hepatoma cell line Huh7 and HepG2, and the mouse hepatoma cell line Hepa1-6 were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % fetal bovine serum (FBS, Gibco, Carlsbad, CA), 100 units/ml penicillin, and 100 mg/ml streptomycin (Thermo Fisher Scientific). The normal mouse hepatocyte cell line AML12 were cultured in DMEM/F-12 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10 % FBS (Gibco, Carlsbad, CA), 5 μg/ml insulin–-transferrin–sodium selenite (ITS) (Life Technologies) containing insulin, transferrin and selenium, 40 ng/ml dexamethasone, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.25 g/L of glutamine as described in the literature (Helsley et al., 2023). All cells were maintained at 37 °C in a humidified atmosphere containing 95 % air and 5 % CO_2. The 10 well-known steatosis inducers (amiodarone hydrochloride, doxycycline hydrochloride, tetracycline hydrochloride, clofibrate, sodium valproate, cyclosporine A, fenofibrate, tamoxifen, tianeptine sodium salt, ticlopidine) and 5 negative controls (sodium citrate, amikacin, colchicine, rotenone, *tert*-butyl hydroperoxide) were purchased from GlpBio (California, USA). ML261 (Cat. No. HY-111179) was purchased from Med Chem Express (MCE, NJ, USA). BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene, Cat. No. D3922) was purchased from Invitrogen (Madrid, Spain). 4′,6-Diamidino-2-phenylindole (DAPI) (Cat. No. D9542) were purchased from Sigma.

Lipid droplet detection by fluorescence microscopy

The Huh7 cells were seeded into 8-well chambers at a density of about 4 x 10^4 cells/mL and cultured with 0.2 ml culture medium each well and allowed to adhere until they reached about 70 % confluence, after treatment and the final incubation, the cells were washed once with Dulbecco's phosphate-buffered saline (D-PBS) and fixed with 4 % paraformaldehyde for 10 min at room temperature, followed by another washed with D-PBS. The cells were then incubated at 37℃ in D-PBS in the dark for 30 min with 2 μM BODIPY 493/503 and 5 μ g/ml DAPI. Next, the cells were washed with D-PBS three times. Lastly, the lipid droplets were immediately visualized using fluorescence microscopy.

Lipid droplet detection by fluorometry

The Huh7 cells were seeded into 96-well black culture plates with a clear bottom (Corning, NY, USA) at a density of about 4 x 10^4 cells/mL and cultured with 0.1 ml culture medium each well, and allowed to adhere until they reached about 70 % confluence. After treatment and the final incubation, the cells were washed once with D-PBS and fixed with 4 % paraformaldehyde for 10 min at room temperature, followed by another D-PBS wash. The cells were then incubated at 37℃ in the dark for 30 min with 2 μM BODIPY 493/503 and 5 μ g/ml DAPI in D-PBS. Next, the cells were washed three times with D-PBS. Lastly, fluorescence intensity was measured by a multimode reader, eplex (Tecan, Austria) at wavelengths of ex 485 nm and em 520 nm for BODIPY 493/503 as well as ex 350 nm and em 450 nm for DAPI. Lipid droplet content was expressed as the relative fluorescence intensity of BODIPY 493/503, normalized to the fluorescence intensity of DAPI in the same sample and then further normalized to the control values.

Cytotoxicity assay

The cytotoxicity of the tested compounds was determined by Cell Counting Kit-8 assay (CCK-8; TransGen, Beijing, China). Briefly, 0.5–1 hour (h) before the end of incubation period, the CCK-8 solution was added into each well of the 96-well plate at a final concentration of 10 %, and mixed gently. The plate was then returned to the incubator immediately. After 0.5–1 h, absorbance (A) was measured at 450 nm with a reference wavelength of 630 nm using the above-mentioned multimode reader. Relative cell viability was determined using the following formula: Relative cell viability = (A450/A630 of an experimental group)/ (A450/A630 of the control group).

Assay precision validation for fluorometry

The precision of the newly established lipid droplet detection assay, fluorometry, was validated by determining the intraplate variability and the consistency with fluorescence microscopy. Intraplate variability was evaluated by treating half the wells in a 96-well plate with 0.15 mM FFA mixture (0.1 mM oleate acid (OA)/0.05 mM palmitate acid (PA) (0.1O/0.05P)), while the remaining wells were treated with the vehicle as control for 24 h. Lipid droplet was then detected by fluorometry, and the relative lipid droplet content between the 0.1O/0.05P group and the control group was calculated. The mean relative value and the coefficient of variation (CV) were calculated. For concomitant evaluation of the consistency, the cells were plated at the same density in 8-well

chambers at the same time and treated identically to the 96-well plate experiment. Lipid droplet detection was performed by fluorescence microscopy for comparison.

Treatment of cultured cells with tested compounds

The Huh7, AML12 cells or Hepa1-6 cells were seeded into 96-well black culture plates with a clear bottom at a density of about 4 x 10^4 cells/mL and cultured with 0.1 ml culture medium each well, and allowed to adhere until they reached about 70 % confluence. For the 10 well-known steatosis inducers and 5 negative controls, Huh7 cells were preincubated with a 63 μM mixture of OA and PA (2:1 ratio) for 14 h, followed by a medium change containing different concentrations of the test compounds or an equal amount of solvent for an additional 24 h as described in the literatures (Donato et al., 2009; Donato et al., 2012). In detail, the working concentration of the chemicals was as bellow: amiodarone hydrochloride (6.25, 12.5, 25 and 50 μM), doxycycline hydrochloride (62.5, 125, 250 and 500 μM), tetracycline hydrochloride (50, 100, 200 and 400 μM), clofibrate (31.2, 62.5, 125 and 250 μM), sodium valproate (1000, 2000, 4000 and 10000 μM), cyclosporine A (10, 20, 50 and 100 μM), fenofibrate (62.5, 125, 250 and 500 μM), tamoxifen (2.5, 5, 10 and 20 μM), tianeptine sodium salt (31.25, 62.5,125 and 250 μM), ticlopidine (25, 50, 100 and 200 μM); sodium citrate (100, 300, 500 and 1000 μM), amikacin (1000, 2500, 5000 and 10000 μM), colchicine (100, 200, 500 and 1000 μM), rotenone (0.1, 0.5, 1 and 5 μM), *tert*-butyl hydroperoxide (5,10,20 and 50 μM). For ML261, AML12 cells and Hepa1-6 cells were co-incubated with a 150 μM mixture of OA and PA (2:1 ratio) and different concentrations of ML261 or solvent for 24 h. In detail, the different working concentration of the ML261 was 0.01, 0.1, 1 and 10 μM. The stock solutions of all the compounds were prepared in dimethyl sulfoxide (DMSO) or water and diluted in the culture medium to obtain the desired final concentrations. The final DMSO concentration in the culture medium did not exceed 0.5 % (v/v).

Screening process for new drugs inhibiting lipid accumulation

Huh7 cells were seeded into 96-well black culture plates with a clear bottom at a density of about 4 x 10^4 cells/mL and cultured with 0.1 ml culture medium each well, and allowed to adhere until they reached about 70 % confluence. After cell adhesion, the cells were incubated with a 150 μM mixture of OA and PA (2:1 ratio) and different concentrations of the test compounds or the equal amount of solvent for 24 h. Then the cytotoxicity was tested by CCK-8 assay above-mentioned, followed by lipid droplet detection by fluorometry as previously described immediately.

Statistical analysis

All values are depicted as the mean ± standard error of measurement (SEM) or mean ± standard deviation (SD) as indicated. Statistical analysis was performed using GraphPad Prism software, version 8.0.1. For comparisons between different groups, Student's *t* test and oneway ANOVA were used for two-group and multiple-group comparisons, respectively. $P < 0.05$ was considered statistically significant. The results shown are representative of at least three independent experiments unless otherwise stated.

Results

Determination of optimal BODIPY 493/503 and DAPI concentrations for fluorometry

Since BODIPY 493/503 has not been previously used for fluorometry, we first determined the optimal concentration of BODIPY 493/503 for this assay. Huh7 cells were treated with 0.3 mM FFA mixture (0.2 mM OA/0.1 mM PA (0.2O/0.1P)) or an equivalent volume of solvent for 24 h (Fig. 1A). Lipid droplet content was detected by fluorometry using BODIPY 493/503 with increasing working concentration (1, 2, 4, 8, and 16 μM), and the cell number was calibrated using protein concentration. The results showed that as the concentration of BODIPY 493/503 increased, the absolute fluorescence intensity increased in both the FFA treatment group and the control group (Fig. 1B). However, the relative fluorescence intensity between the FFA treatment group and control groups did not change significantly as the concentration of BODIPY 493/503 increased (Fig. 1C). Therefore, 2 μM was selected as the optimal concentration for BODIPY 493/503, as it provided a sufficient fluorescence intensity (greater than 10,000 and at least two orders of magnitude higher than the blank wells) at a relatively low concentration to avoid possible background. Next, we determined the optimal concentration of DAPI. Similarly, Huh7 cells were treated with FFAs (0.2O/0.1P) or an equal dose of solvent for 24 h. Then, lipid droplet content was detected by fluorometry using BODIPY 493/503 at 2 μM, and cell numbers were calibrated using DAPI with increasing working concentrations (2.5, 5, 10, 20, and 40 μg/ml). The results showed that as the concentration of DAPI increased, the absolute fluorescence intensity of it also increased in both the FFAs treatment group and the control group (Fig. 1D). However, compared with the control group, the relative fluorescence intensity of BODIPY 493/503 in the FFAs treatment group did not change with increasing DAPI concentration (Fig. 1E). Therefore, 5 μg/ml was determined as the optimal concentration for DAPI, as it provided a sufficient fluorescence intensity (greater than 10,000 and at least two orders of magnitude higher than the blank wells) at a relatively low concentration to avoid possible background. Therefore, in subsequent experiments, 2 μM BODIPY 493/503 and 5 μg/ml DAPI were selected.

Influence of cell fixation and CCK-8 on lipid droplet detection by fluorometry using BODIPY 493/503

BODIPY 493/503 is known to stain both fixed and unfixed cells. We next evaluated the impact of fixation on our fluorometry assay. Huh7 cells were treated with different concentrations of FFAs (0.1P, 0.2O/0.1P, 0.4O/0.2P) or equal amount of solvent for 24 h (Fig. 2A). The fluorometry assay results showed that in the fixed cells, lipid droplet content increased in a dose-dependent manner in response to FFAs treatment, compared to the control group. However, in unfixed cells, lipid droplet content did not increase with higher concentration of FFAs and remained similar to the control group (Fig. 2B). These results indicated that cell fixation is necessary for BODIPY 493/503 staining in fluorometry.

CCK-8 is a safe, non-toxic assay for detecting cell viability. In order to save costs and improve efficiency, we planned to first use CCK-8 to detect drug toxicity, followed by fluorometry to detect the effect of these drugs on lipid deposition in the same 96-well plate. We further evaluated the effect of CCK-8 on our fluorometry assay. Huh7 cells were treated with different concentrations of FFAs (0.1O/0.05P, 0.2O/0.1P, 0.2O/0.2P, 0.4O/0.2P) or an equivalent amount of solvent for 24 h (Fig. 2C). The results of fluorometry showed the FFA treatment group showed a dose-dependent increase in lipid droplet content compared to the control group, regardless of whether CCK-8 was used. Although the absolute fluorescence values of BODIPY 493/503 and DAPI changed with CCK-8 solution treatment, the ratio of the two was not affected (data not provided), and compared with the control group, the relative fluorescence intensity of BODIPY 493/503 in the FFA treatment group was also not affected (Fig. 2D). These results indicated that it is feasible to use CCK-8 to measure cell viability before detecting lipid droplet content via fluorometry using BODIPY 493/503.

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O
B	H_2O	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	H_2O
C	H_2O	Con	0.2O/0.1P	Con	0.2O/0.1P	Con	0.2O/0.1P	Con	0.2O/0.1P	Con	0.2O/0.1P	H_2O
D	H_2O	Con	0.2O/0.1P	Con	0.2O/0.1P	Con	0.2O/0.1P	Con	0.2O/0.1P	Con	0.2O/0.1P	H_2O
E	H_2O	Con	0.2O/0.1P	Con	0.2O/0.1P	Con	0.2O/0.1P	Con	0.2O/0.1P	Con	0.2O/0.1P	H_2O
F	H_2O	Con	0.2O/0.1P	Con	0.2O/0.1P	Con	0.2O/0.1P	Con	0.2O/0.1P	Con	0.2O/0.1P	H_2O
G	H_2O	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	H_2O
H	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O

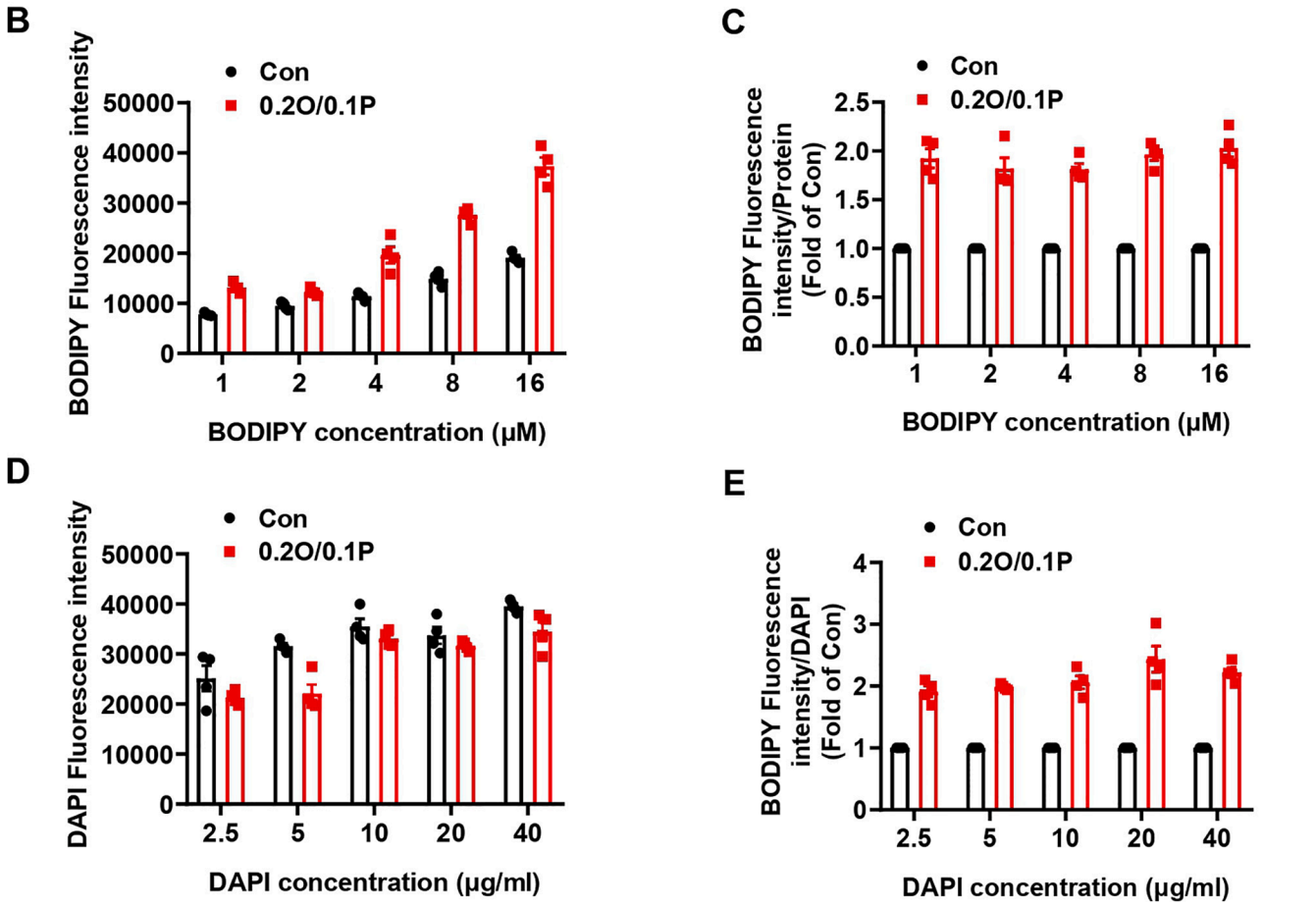

Fig. 1. Determination of the optimal BODIPY 493/503 and DAPI concentrations for fluorometry. (A) Schematic of Huh7 cells treatment with free fatty acids (FFAs) or solvent. Huh7 cells were treated with a 0.3 mM FFA mixture (0.2 mM oleate acid/0.1 mM palmitate acid (0.2O/0.1P)) or an equivalent amount of solvent for 24 h-. Con: solvent-treated cells; 0.2O: 0.2 mM OA; 0.1P: 0.1 mM PA; 0.2O/0.1P: a FFA mixture of 0.2 mM OA with 0.1 mM PA. (B) Absolute fluorescence intensity of BODIPY 493/503 in the FFAs treatment group and the control group. n = 4. Data are presented as mean ± SEM. (C) Relative fluorescence intensity of BODIPY 493/503 in the FFAs treatment group and control group. Lipid droplet content was expressed as relative fluorescence intensity of BODIPY 493/503, normalized to the protein concentration and then to the control values. n = 4. Data are presented as mean ± SEM. (D) Absolute fluorescence intensity of DAPI in the FFAs treatment and control group. n = 4. Data are presented as mean ± SEM. (E) Relative fluorescence intensity of BODIPY 493/503 in the FFAs treatment and control group. Lipid droplet content was expressed as relative fluorescence intensity of BODIPY 493/503, normalized to the fluorescence intensity of DAPI in the same sample and then to the control values. n = 4. Data are presented as mean ± SEM.

Fluorometry provides comparable but more accurate quantitative results than fluorescence microscopy

To evaluate the precision of our fluorometry assay, as described in section 2.5 of Materials and Methods, we treated 96-well plate or 8-well chamber Huh7 cells with FFAs (0.1O/0.05P) or an equivalent amount of solvent for 24 h (Fig. 3A). Lipid droplets were then quantified using both fluorometry or fluorescence microscopy. The results of fluorometry assay showed a significant increase in lipid droplet content in FFAs treatment group compared to the control group, with an average increase of 1.65 folds (SD = 0.063, CV = 3.83 %) (Fig. 3B). The results of fluorescence microscopy showed the content of lipid droplets in FFAs

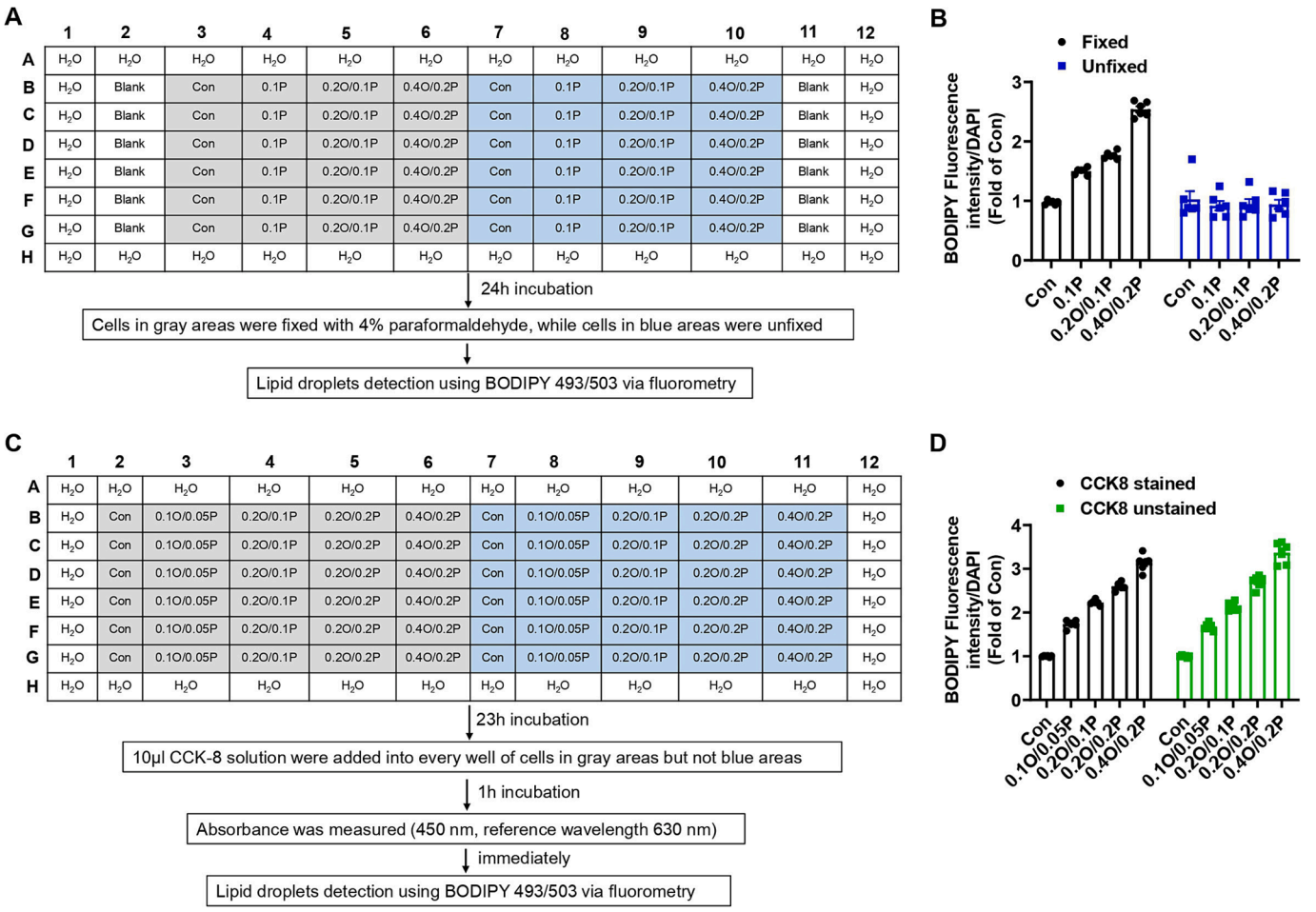

Fig. 2. Lipid droplet detection by fluorometry using BODIPY 493/503 influenced by unfixing but not CCK-8. (A) Schematic of the Huh7 cells treatment with FFAs or solvent to evaluate the impact of fixation on our fluorometry assay. (B) Relative fluorescence intensity of BODIPY 493/503 in the FFAs treatment and control group. Lipid droplet content was expressed as the relative fluorescence intensity of BODIPY 493/503, as described in Fig. 1E. n = 6. Data are presented as mean ± SEM. (C) Schematic of the treatment of Huh7 cells with FFAs or solvent to evaluate the effect of CCK-8 on our fluorometry assay. (D) Relative fluorescence intensity of BODIPY 493/503 in the FFAs treatment and control group. Lipid droplet content was expressed as the relative fluorescence intensity of BODIPY 493/503, as described in Fig. 1E. n = 6. Data are presented as mean ± SEM.

treatment group was also significantly increased, compared to the control group, with an average increase of 1.66 folds (SD = 0.17, CV = 10.42 %) (Fig. 3C-D). To further verify the consistency of the two methods, we treated 8-well chamber Huh7 cells with another concentration of FFAs (0.2O/0.1P) or an equivalent amount of solvent for 24 h and lipid droplets were then quantified using fluorescence microscopy. The results showed that compared with the control group, the lipid droplet content in the 0.2O/0.1P group was significantly increased with an average increase of 2.11 folds (Fig. S1A-B), which was similar to the results of Huh7 cells in the 0.2O/0.1P group showed in Fig. 4C, which was detected by the fluorometry assay with an average increase of 2.04 folds. These results indicated the quantification of lipid droplets by fluorometry is comparable to that of fluorescence microscopy but offers greater precision, as reflected by the lower variability in the fluorometry assay.

Determination of appropriate concentration of FFAs for drug screening

FFA induced hepatocyte lipid deposition is an widely used *in vitro* model for studying steatosis (Gómez-Lechón et al., 2007). In order to favor and accelerate lipid accumulation and the development of steatosis-associated cell injury, a previous study pretreated cells 14 h with 0.062 mM FFAs (OA/PA, 2:1) before drug treatment to evaluate drug-induced steatosis (Tolosa et al., 2018). In this study, we also intended to pre-treat cells with FFAs for 14 h when validating the fluorometry assay with the 15 selected well-known steatosis inducers and negative controls. Additionally, we planned to utilize FFAs for simultaneous incubation when assessing the inhibitory impact of ML261 on steatosis. Therefore, we first determined the appropriate concentration of FFAs. Huh7 and HepG2 cells were treated with different concentrations of FFAs (0.042O/0.021P, 0.1O/0.05P, 0.2O/0.1P, 0.4O/0.2P) or equal doses of solvent as control for 24 h (Fig. 4A). The CCK-8 assay results showed that when the FFAs concentration was 0.042O/0.021P, there was no significant toxicity to both Huh7 and HepG2 cells. Starting from 0.1O/0.05P, there was a significant dose-dependent toxicity observed; At the highest concentration, HepG2 cells exhibit greater toxicity than Huh7 cells (Fig. 4B). The fluorometry assay results showed that compared with the control group, when the FFAs concentration was 0.042O/0.021P, the lipid content of HepG2 cells but not Huh7 cells showed a significant increase. Starting from 0.1O/0.05P, both Huh7 cells and HepG2 cells showed a significant dose-dependent increase in lipid content (Fig. 4C). Based on these results, this study chose 0.042O/0.021P for FFAs pretreatment prior to drug treatment in assessing drug-induced steatosis, and 0.1O/0.05P for simultaneous incubation to evaluate the effect of drugs on lipid deposition. Huh7 cells were selected for subsequent validation experiments.

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O
B	H_2O	Con	Con	Con	Con	Con	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	H_2O
C	H_2O	Con	Con	Con	Con	Con	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	H_2O
D	H_2O	Con	Con	Con	Con	Con	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	H_2O
E	H_2O	Con	Con	Con	Con	Con	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	H_2O
F	H_2O	Con	Con	Con	Con	Con	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	H_2O
G	H_2O	Con	Con	Con	Con	Con	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	0.1O/0.05P	H_2O
H	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O

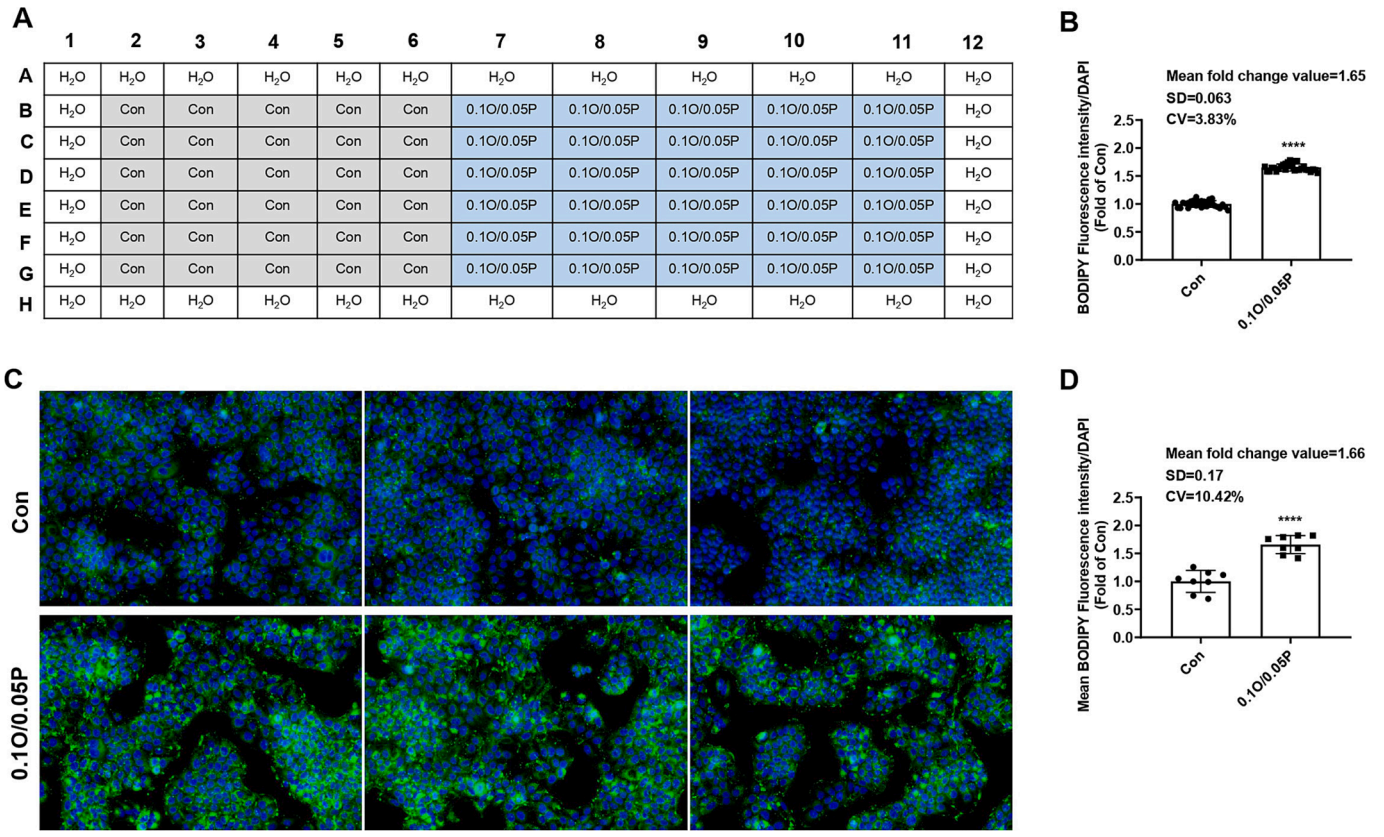

Fig. 3. The quantitative results of fluorometry are comparable to fluorescence microscopy, with higher precision. (A) Schematic of Huh7 cells treatment with FFAs or solvent to test the precision of our fluorometry assay. (B) Relative fluorescence intensity of BODIPY 493/503 in the FFAs treatment and control group. Lipid droplet content was expressed as the relative fluorescence intensity of BODIPY 493/503, as described in Fig. 1E. SD: standard deviation; CV: coefficient of variation. n = 30. Data are presented as mean ± SD. ****$P < 0.0001$ vs. Con cells. (C-D) Huh7 cells were treated with 0.1O/0.05P or equivalent amount of solvent for 24 h. Intracellular lipid droplet levels were measured using BODIPY 493/503 and detected by fluorescence microscopy. Representative images were shown in (C) with green representing BODIPY 493/503-stained lipid droplet and blue indicating DAPI-stained nuclei; Quantitative data are shown in (D). n = 8. Data are presented as mean ± SD. ****$P < 0.0001$ vs. Con cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Validation of fluorometry assay using known steatosis inducers and negative controls

To validate the utility of our newly established fluorometry assay, we selected 10 known steatosis inducing drugs and 5 negative controls for validation. The working concentrations of these 15 drugs were selected based on published literatures (Donato et al., 2009; Donato et al., 2012). Huh7 cells were treated as described in Materials and Methods section 2.6. The CCK-8 experiment results showed that all 10 positive drugs exhibited varying degrees of cytotoxicity within the selected working concentration range (Fig. S2A-J). The fluorometry assay results showed a dose-dependent increase in lipid droplet content in all 10 positive drugs exhibited, compared with the control group (Fig. 5A-J), which were consistent with the literatures (Donato et al., 2009; Donato et al., 2012). The CCK-8 results showed citrate and amikacin had no cytotoxicity, while colchicine and rotenone had significant toxicity even at the lowest concentration. Meanwhile, *tert*-butyl hydroperoxide exhibited significant cytotoxicity only at the highest concentration (Fig. S3A-E). The fluorometry assay results showed that all 5 negative drugs had no significant effect on lipid droplet content, compared with the control group (Fig. 6A-E), which were also consistent with the literatures (Donato et al., 2009; Donato et al., 2012). These findings demonstrated that our fluorometry assay accurately identified the effects of the 15 tested drugs on lipid droplet content and cytotoxicity.

Further validation of the fluorometry assay using a known hepatic lipid droplets formation inhibitor

ML261 is an inhibitor of hepatic lipid droplet formation, as reported by Jiwen Zou et al. (Zou et al., 2010) They showed that ML261 effectively inhibits lipid droplet formation in mouse liver cell lines (AML12 cells), with minimal impact on human derived liver cells. To further validate whether our newly established fluorometry assay can be used to screen lipid accumulation inhibitor, we treated AML12 cells as described in Materials and Methods sections 2.6 and 2.7. The CCK-8 experiment results showed that ML261 had no significant cytotoxicity within the working concentration range of 0.01–10 μM (Fig. S4A); The fluorometry assay results showed that ML261 could dose dependently reduce lipid droplet content, compared with the control group (Fig. 7A). We reproduced this assay in another mouse derived cell line, Hepa1-6 cells (Fig. S4B, Fig. 7B), and we obtained consistent results using fluorescence microscopy in Hepa1-6 cells after the same treatment (Fig. S5A-B). These results indicated that our drug screening system based on fluorometry assay is effective and reliable for identifying inhibitors of lipid accumulation.

Discussion

In this study, we developed a cell-based 96-well plate assay to screen potential drugs for inhibiting lipid deposition. In this assay, Huh7 cells were co-incubated with FFAs (0.1O/0.05P) and the compounds of interest (or equal dose of solvent) for 24 h. Then CCK-8 solution was added

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O
B	H_2O	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	H_2O
C	H_2O	Con	0.042O/0.021P	0.1O/0.05P	0.2O/0.1P	0.4O/0.2P	Con	0.042O/0.021P	0.1O/0.05P	0.2O/0.1P	0.4O/0.2P	H_2O
D	H_2O	Con	0.042O/0.021P	0.1O/0.05P	0.2O/0.1P	0.4O/0.2P	Con	0.042O/0.021P	0.1O/0.05P	0.2O/0.1P	0.4O/0.2P	H_2O
E	H_2O	Con	0.042O/0.021P	0.1O/0.05P	0.2O/0.1P	0.4O/0.2P	Con	0.042O/0.021P	0.1O/0.05P	0.2O/0.1P	0.4O/0.2P	H_2O
F	H_2O	Con	0.042O/0.021P	0.1O/0.05P	0.2O/0.1P	0.4O/0.2P	Con	0.042O/0.021P	0.1O/0.05P	0.2O/0.1P	0.4O/0.2P	H_2O
G	H_2O	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	H_2O
H	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O

Gray areas :Huh7 cells Blue areas :HepG2 cells

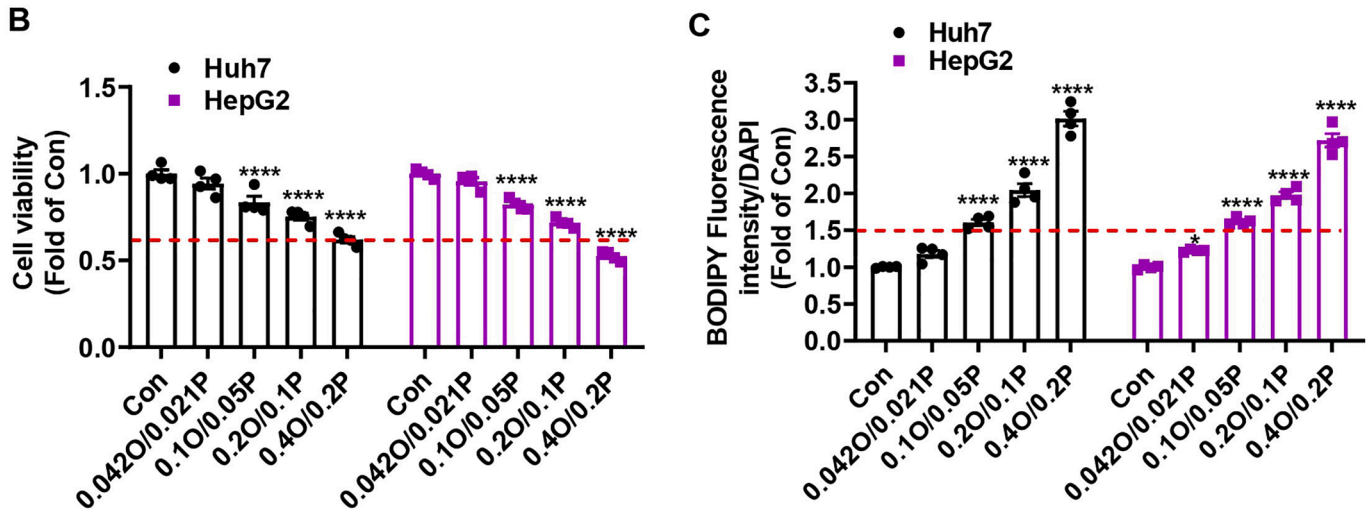

Fig. 4. Cytotoxicity and Fat overloading of hepatic cells with different concentrations of FFAs. (A) Schematic of Huh7 and HepG2 cells treatment with FFAs or solvent to determine the appropriate concentration of FFAs for drug screening. Huh7 and HepG2 cells were treated with different concentrations of FFAs (0.042O/0.021P, 0.1O/0.05P, 0.2O/0.1P, 0.4O/0.2P) or solvent as control for 24 h. Then cytotoxicity was tested via CCK-8 assay and fat overloading was assessed using BODIPY 493/503 through fluorometry. (B-C) Cytotoxicity of the FFAs treatment groups and the control group were shown in (B) and the fat overloading in (C). n = 4. Data are presented as mean ± SEM. $^*P < 0.05$, $^{****}P < 0.0001$ vs. Con cells.

1 h before the end of incubation, and cell viability was detected 1 h later using the CCK-8 assay. Then, the fluorescent probe BODIPY 493/503 was used to detect lipid accumulation by fluorometry assay, and DAPI staining was employed to calibrate cell numbers. To our knowledge, this study is the first to confirm that BODIPY 493/503 can be reliably used for fluorometry assay, and to demonstrate that the use of CCK-8 does not interfere with the subsequent relative quantitation of lipid droplets by BODIPY 493/503.

BODIPY 493/503 has been widely used in fluorescence microscopy and flow cytometry, but there have been no reports of its application in fluorometry assays. Donato et al. reported a cell-based *in vitro* screening methods for evaluating drug-induced steatosis using BODIPY 493/503 via high content screening (HCS) technology (Donato et al., 2012). While this method can efficiently evaluate four toxicity endpoints of drugs, including cell viability, intracellular lipid accumulation, mitochondrial membrane potential, and hydrogen peroxide generation, it relies on expensive instruments. In addition, based on the fluorescence images presented in the article and our own experimental experience, images of certain regions captured by fluorescence microscopy may not fully represent the overall lipid deposition, and image analysis can be time-consuming. They also reported an *in vitro* screening method using BODIPY 493/503 fluorescent dye combined with flow cytometry (Donato et al., 2009). This method effectively eliminates the interference of dead cells on fluorescence through gating, but it requires digestion of adherent hepatocytes, making the sample preparation process cumbersome and time-consuming. Compared to fluorescence microscopy and flow cytometry, fluorometry is more suitable for high-throughput screening due to its ability to reflect the overall lipid deposition, simplified sample preparation, straightforward data analysis, and independence from expensive instruments. Although some fluorometry based methods for evaluating drug-induced steatosis exist, they rely on dyes like Nile Red (Sugarman et al., 2014; Lee et al., 2022), AdipoRed (Alarcan et al., 2021), or BODIPY558/568 C_{12} (Fujimura et al., 2009) to quantify lipids. BODIPY 493/503, however, offers greater specificity, lower background staining, and a narrower emission spectrum, making it an ideal fluorophore for multi label experiments (Durandt et al., 2016; Strauss et al., 2020). In this study, we confirmed that the results of lipid quantification using BODIPY 493/503 via fluorometry were comparable to those of fluorescence microscopy, with even smaller intra group variability, indicating its higher precision. More importantly, our newly established fluorometry method accurately identified 15 known steatosis-positive and −negative inducers, as well as the inhibitory effect of ML261 on lipid accumulation. When we tested these 15 known steatosis-positive and −negative drugs, the drugs detected on different 96-well plates were not conducted blindly, but they were randomly selected according to category number, thus reducing the potential bias that may be introduced by non-blindness as much as possible. These findings indicate the precision and practicality of this method for drug screening.

In this study, we confirmed that after detecting cell viability with CCK-8 in the same 96-well plate, lipid accumulation can still be detected using BODIPY 493/503. And CCK-8 does not affect the relative

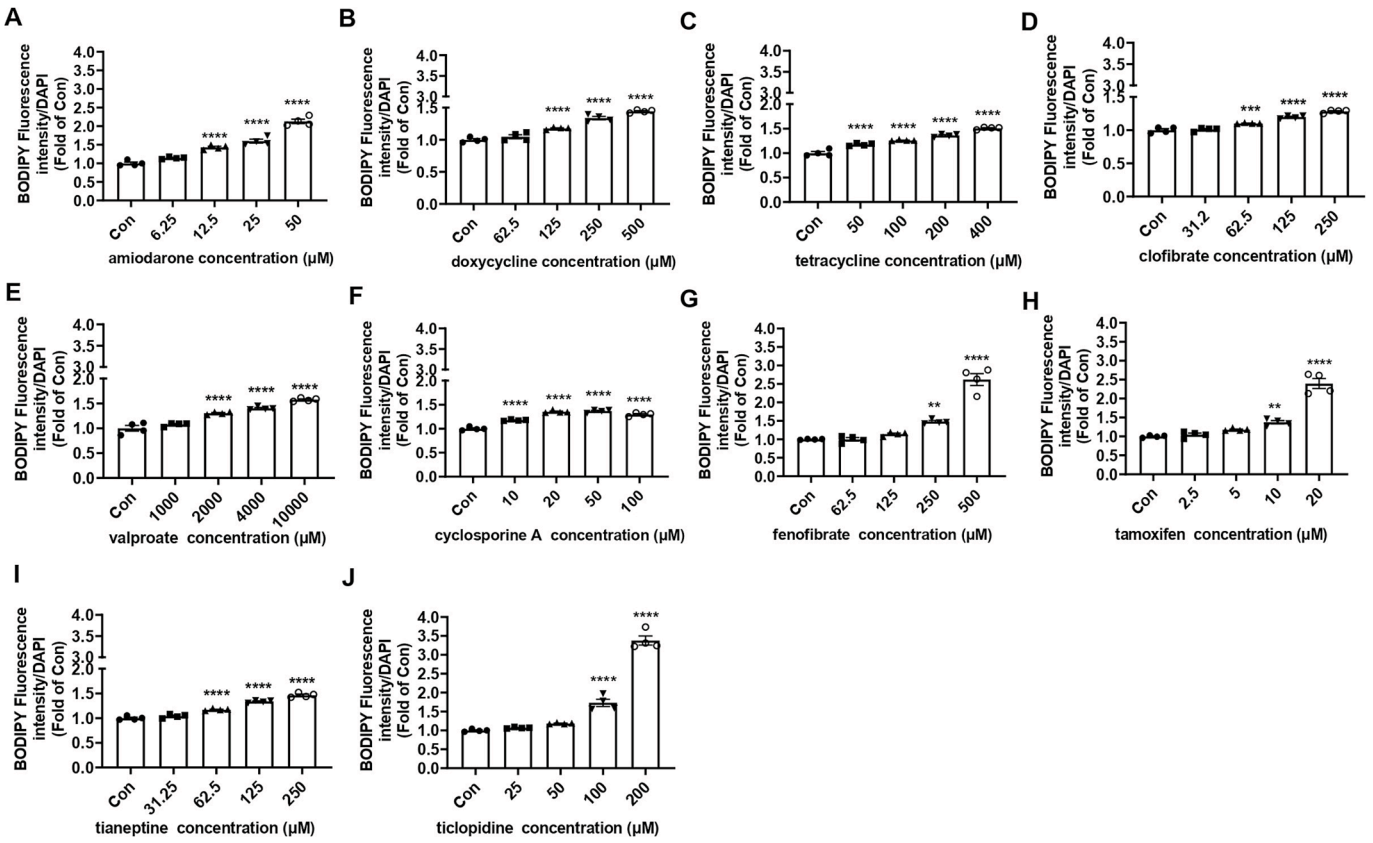

Fig. 5. Fat accumulation assessment of concentration-dependent effects of steatogenic compounds on Huh7 cells. (A-J) Huh7 cells were treated as described in Materials and Methods section 2.6, and fat accumulation were assessed using BODIPY 493/503 via fluorometry assay. The effects of amiodarone hydrochloride (A), doxycycline hydrochloride (B), tetracycline hydrochloride (C), clofibrate (D), sodium valproate (E), cyclosporine A (F), fenofibrate (G), tamoxifen (H), tianeptine sodium salt (I), and ticlopidine (J) on fat accumulation were displayed as the relative fluorescence intensity of BODIPY 493/503, as described in Fig. 1E. All 10 positive drugs exhibited a dose-dependent increase in lipid droplet content. n = 4. Data are presented as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ vs. Con cells.

quantification results of BODIPY 493/503 on lipids. This allows our drug screening system to simultaneously evaluate the effects of drugs on both cell toxicity and lipid deposition, without the need for a parallel plate, significantly saving time and costs.

FFAs induced lipid deposition in hepatocytes is an ideal *in vitro* model for studying steatosis, but FFAs also have cytotoxicity, making it essential to choose an appropriate concentration. In this study, we tested a series of FFAs concentrations (0.042O/0.021P, 0.1O/0.05P, 0.2O/0.1P, 0.4O/0.2P) to evaluate their cytotoxicity and the ability to induce of lipid deposition. The results showed that at 0.042O/0.021P, there was no significant change in cell viability of Huh7 cells and HepG2 cells, compared with the control group. The lipid deposition of Huh7 cells showed no statistically significant increase, while that of HepG2 cells showed a slight statistically significant increase. At higher concentrations, 0.1O/0.05P to 0.4O/0.2P, both cell lines showed a significant dose-dependent decrease in cell viability and a significant dose-dependent increase in lipid deposition compared to the control group. Therefore, in this study, we chose Huh7 cells to validate 15 known steatosis-positive and −negative inducers, and as reported in previous studies (Donato et al., 2009; Donato et al., 2012), 0.042O/0.021P was pre incubated for 14 h before drug treatment, because this concentration of FFAs treatment did not cause significant cytotoxicity but could promote lipid deposition. ML261 was found to significantly affect lipid deposition only in mouse derived hepatocytes (Zou et al., 2010). Therefore, to evaluate the inhibitory effect of ML261 on lipid deposition, AML12 cells and Hepa1-6 cells were co-incubated with different concentrations of ML261 (or equal dose of solvent) and FFAs at the concentration of 0.1O/0.05P for 24 h. Although this FFAs concentration had notable cytotoxicity, cell viability remained above 80 %, while inducing sufficient lipid accumulation (fold change value greater than 1.5).

High throughput is crucial for drugs screening. In our newly established method, a cell-based 96-well plate format is adopted. For initial screening of unknown compounds, which are typically soluble in water or DMSO, water or DMSO can be used as the solvent control. This setup allows for the simultaneous testing of at least 18 compounds per a 96 well plate, with three replicates for each treatment. Although this method currently uses a 96-well format, it could potentially be adapted to 384-well or 1536-well plates, though further experimental validation is needed. In addition, our newly established method can also be used for lipid quantification in cells other than hepatocytes, such as cardiomyocytes (data not provided).

In summary, this study demonstrates the successful establishment of a simple and reliable method for screening potential inhibitors of fatty liver. The assay does not require expensive instruments and is easy to implement. While it does not provide comprehensive liver toxicity parameters, it can quickly and efficiently access both the cytotoxicity of drugs and their impact on lipid deposition in the early stages of drug development. This makes it a valuable tool for identifying drug candidates for the treatment of NAFLD.

CRediT authorship contribution statement

Weili Yang: Conceptualization, Methodology, Formal analysis, Writing – original draft, Funding acquisition. **Qiuyue Pan:** Methodology. **Qi Li:** Methodology. **Sirui Zhou:** Methodology. **Xi Cao:** Formal analysis, Funding acquisition, Writing – review & editing.

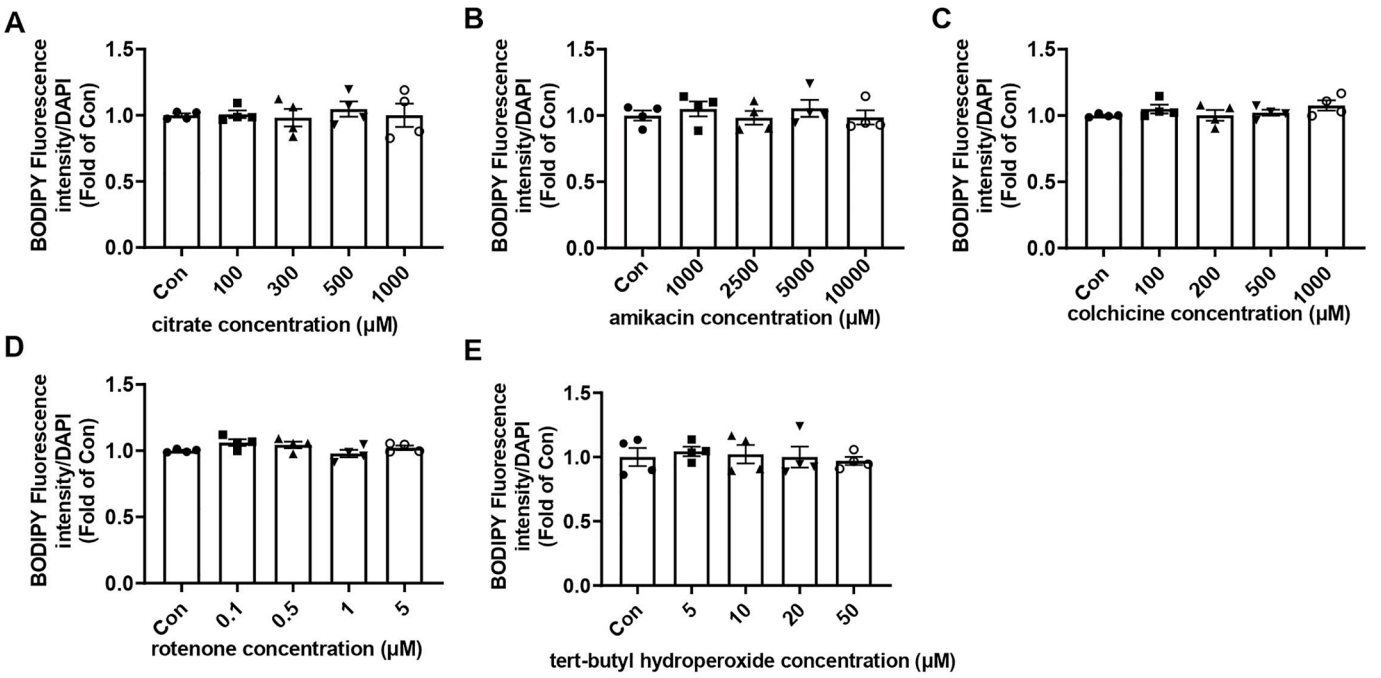

Fig. 6. Fat accumulation testing of nonsteatotic compounds in Huh7 cells. (A-E) Huh7 cells were treated as described in Materials and Methods section 2.6, and fat accumulation were assessed using BODIPY 493/503 via fluorometry assay. The effects of sodium citrate (A), amikacin (B), colchicine (C), rotenone (D), and *tert*-butyl hydroperoxide (E) on fat accumulation were displayed as the relative fluorescence intensity of BODIPY 493/503, as described in Fig. 1E. None of these 5 negative drugs had a significant effect on lipid droplet content. n = 4. Data are presented as mean ± SEM.

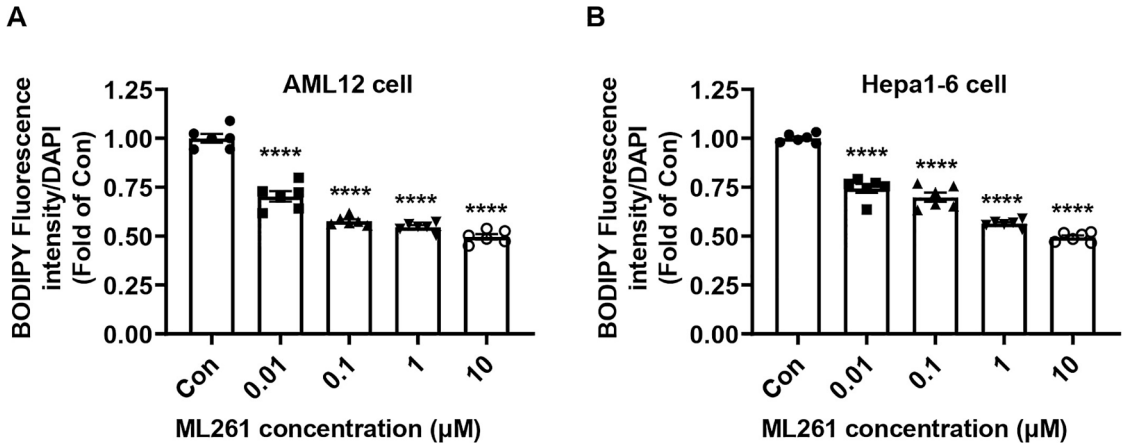

Fig. 7. The newly established fluorometry method correctly identified the inhibitory effect of ML261 on lipid accumulation. (A-B) AML12 cells and Hepa1-6 cells were treated as described in Materials and Methods section 2.6 and 2.7. The effects of ML261 on fat accumulation of AML12 cells (A) and Hepa1-6 cells (B) were displayed as the relative fluorescence intensity of BODIPY 493/503, as described in Fig. 1E. ML261 dose-dependently reduced lipid droplet content in both AML12 cells and Hepa1-6 cells. n = 6. Data are presented as mean ± SEM. ****$P < 0.0001$ vs. Con cells.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2024.100213.

Data availability

Data will be made available on request.

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