Summary of the Ph.D. Thesis

Development of novel dyes and fluorescence-imaging based approaches for cellular localization and dynamics of lipid droplets

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Introduction

Plant cells store neutral lipids in distinct cytosolic organelles called lipid droplets (LDs). LDs are made up of a neutral lipid core surrounded by a phospholipid monolayer, with a series of proteins embedded in their surface. LD biogenesis in plants is thought to occur in the ER, where they are assembled in specialized ER microdomains. In the past, LDs were mostly regarded as static carbon depots found only in oleogenic seeds. But in recent years evidence has emerged pointing towards the presence of dynamic LDs in different cell types and also broadening their role in plants including, playing a role in various processes such as stress response, pathogen resistance, lipid homeostasis, hormone metabolism and signaling. But much is still unknown about this dynamic organelle, like its transport mechanism, mechanism of protein targeting, assembly and regulation. Furthermore, the neutral lipid content (contained mostly in LDs) of cells has been linked to cell cycle progression. Earlier work has shown that the intensity of neutral lipids of MA-10 Leydig tumor cells labelled with BODIPY 493/503, was a function of the cell cycle. Neutral lipid biosynthesis has also been reported to be coordinated with cell cycle progression in dinoflagellates. Taking the above into account, live cell analysis would be necessary to unravel the dynamic role of LDs in plant cells.

In recent years, there have been particular advances in the use of live imaging systems with the emergence of a variety of novel imaging tools. Combining a reliable LD marker with these tools can create a versatile scheme for exploring LD biology. However, many commercially available live cell LD dyes such as Nile Red and BODIPY 493/503 have certain drawbacks such as broad emission range and lesser photostability. Other vital LD dyes with better photostability, such as BODIPY 505/515 and LD540, still fluoresce in the green to orange region of the visible spectrum, hindering multi-color imaging. Therefore, the identification of a vital dye that can stain LDs while being spectrally well separated from most green, orange and red fluorescence reporters will be a great asset for live cell imaging in studying LD biology. However, designing a fluorochrome with a specific target in mind (e.g. LD localizing dyes) is a tedious and expensive process. Through the course of this work, a method was developed to combine small molecule microarray (SMM) prescreening and confocal laser scanning microscopy (CLSM) in order to discover novel cell staining fluorescent dyes.

Main objectives of the thesis

The goals of this work are as follows:

- Characterizing the potential novel fluorochromes in human HeLa cells and potential LD fluorochromes in several plant cell suspension cultures.

- Analyzing their intracellular labeling, spectral properties, optimal working concentration and assessing their toxicity to confirm their use for long-term live cell analysis.

- Employing novel EdU based S-phase cell cycle detection in combination with LD dyes to study LD variability during cell cycle.

Methods

- Chemicals screened: The 14,585-member small molecule collection screened during the discovery of eleven live cell staining fluorescent dyes, consisted of two commercially available libraries: 8,800 compounds from Nanosyn (Santa Clara, CA, USA) and 5,120 compounds from Enamine (Kiev, Ukraine), and a 665-member library of Avidin Ltd. (Szeged, Hungary). The three chemicals characterized as plant lipid droplet dyes, Ac-201, Ac-202 and Ac-1041, were in-house synthesized thalidomide analogs.
- Treatment of human HeLa cells with selected compounds: One day after plating, cell cultures were treated with the chemicals at 1 and 10 μM for 1–2 h or one day. Photoconversion of chemical C2 was achieved either via successive scanning with 633 nm HeNe laser at 50% laser intensity setting or using the rhodamine filter set excitation (510–550 nm) for 10 s using maximum intensity of HBO 103W/2 mercury short arc burner. Stability of chemical C2 at low laser intensity was recorded using successive scanning with 633 nm laser at 4% laser intensity setting. Control cultures were treated with 1% DMSO.
- Plant cell culture treatment with fluorochromes: The cell cultures were treated with the chemicals at 1, 10 and 50 μM for various durations. Dye labeling of Arabidopsis seedlings were done using 10 μM Ac chemical for 1 h. LD540 dye was used at 0.3 ng/mL for 15 min. DMSO (1 %, v/v) was used as carrier control in all experiments.

- Colocalization: Selected chemicals were co-labeled with known fluorescent markers on HeLa cells and plant suspension cultures.
- EdU labelling of BY-2 cells: BY-2 cells were labelled with 10 μM final concentration of EdU for 30 minutes. Cells were washed once in PBS and fixed for 30 minutes in 4% PFA.
- Blocking BY-2 cells in S-phase using hydroxyurea (HU): 5-day old BY-2 culture refreshed and blocked immediately with 5mM HU, for 36 hours. HU block was removed by washing the cells with the sterile supernatant of a 36-hour old culture and further cultured in the same medium. BY-2 and control cells before and 2 hours after wash were labelled with Ac-201 at 10 μM concentration for 15 minutes before image acquisition.
- Confocal laser scanning microscopy is used for image acquisition.
- 3D image analysis and LD quantification were performed using Huygens Professional software.

Results

1. Small molecule microarray prescreened compounds were first screened for fluorescence intensity, cell permeability, solubility and toxicity under *in vivo* conditions in human HeLa cells using confocal laser scanning microscopy (CLSM). The analysis of the compounds was performed with all four available lasers of the CLSM (405, 488, 543 and 633 nm). Chemicals which were cell impermeable, toxic, displaying low solubility or fluorescence emission were eliminated and in the process we have found five chemicals localized to lipid droplets, three to mitochondria, one to plasma membrane, one to perinuclear/cytoplasmic region and one to mitochondria/nucleolus.
2. All of the chemicals were labeling the target compartment within one hour of incubation at 37 °C when used at 10 μM concentration. Chemical C2 was not well tolerated by the cells at 10 μM, however at concentrations as low as 0.25 μM, its signal was very bright and detectable. Therefore, its use was limited to a maximum of 1 μM. We also tested the stability and labeling persistency of these chemicals over time. Apart from chemicals E5, D10 and C3, they were still fluorescent even after one day of incubation at 37 °C, proving their use for long-term live cell analysis.

3. All 11 autofluorescent compound's intracellular localization was determined by colocalization with known fluorescent markers. DNA dye RedDot 1 was co-labeled with chemical B5, which is localized to the perinuclear region. Oil Red O was used for staining of LDs colocalizing with chemicals C4, C6, B2, B3 and B4 on fixed HeLa cells. Chemicals D10, E5, C2 and C3 were all localized to mitochondria. Red-colored D10 and E5 were co-stained with green-colored NAO and deep-red colored chemicals C2, C3 with red-colored MitoTracker Orange. All four resulted in complete colocalization with these mitochondria localized markers. Chemical B11 was found to localize preferentially to plasma membrane. The whole cell-labeling FDA was co-stained with chemical B11 to confirm its localization.
4. We tested the effect of these chemicals on long-term viability of cultured cells. 4-hydroxytamoxifen (HT), a known apoptosis inducer was used at a concentration of 20 μM, as a positive control. During a 24-hour period, HeLa cells were treated with chemicals at a concentration of 10 μM (1 μM for chemicals C2 and its photoconverted form, C2*). Viability was assessed using FDA staining. As compared to DMSO-treated cells, 24-hour treatment with the chemicals did not significantly affect cell viability, except C2* and HT. Hence, we inferred that these dyes are also suitable for long-term labeling and tracking experiments. Although C2* did not induce any visible morphological changes during 1-hour post conversion, 24h treatment significantly caused cell round-up with reduced viability. Therefore, we concluded that the application of C2* is limited to short term colocalization analyses.
5. The chemicals were also tested for apoptotic nuclei formation as an alternative approach. Cells treated with HT at 20 μM and with compounds at 10 μM (1 μM for C2 and C2*) for 24 h were fixed and stained with DAPI to screen nuclear morphology changes. None of the chemicals displayed nuclear abnormalities except C2* and HT for which blebs and micronuclei were frequently observed.
6. Of the five LD localizing dyes discovered, only three, B2, B3 and C4 were found to penetrate the cell wall and displayed bright, blue, LD like spotty signals. However, these novel dyes were available in limited amount and hence were not further pursued as potential LD dyes for plant studies. Ac chemicals, Ac- 201, Ac-202 and Ac-1041, which were more abundant were used in our following studies.

7. All three chemicals penetrated cells of rice suspension cultures (*Oryza sativa* L. japonica cv. 'Unggi 9') and displayed bright spotty signals. For Ac-201 and Ac-202 spotty signals were detectable at 1 μM and 15 min incubation but Ac-1041 labeling was weak at this concentration. For long-term experiments 1 μM concentration was not sufficient. Higher concentration treatments of 10 and 50 μM resulted in faster accumulation of bright signals within 15 min. Ac-201 and Ac-202 were still visible after 24-hours when used at 10 μM concentration. It was observed that Ac-202 showed the brightest signal in the rice culture used in our experiments and at least 10 μM concentration was needed for proper detection of Ac-1041 chemical in short-term experiments. Control cultures treated with 1 % (v/v) of solvent DMSO did not display detectable blue autofluorescence in short-term or long-term experiments.
8. We have incubated Ac chemical-treated cells with propidium iodide (PI) to assess the effect of these chemicals on cell viability and plasma membrane integrity. No significant increase in PI labeling was observed with the use of Ac chemicals at 1–50 μM concentration range, even after 24-hour treatment. Bright field microscopy images were also comparable to control, DMSO-treated cultures.
9. In control experiments, at 1 % DMSO concentration, cell viability was not affected significantly. However, significant increase in cell mortality of cultured cells was seen at 10 % (v/v) DMSO, evident even after 15 min. Untreated young cultures showed low level of cell death, similar to 1 % DMSO-treated cultures. As the culture gets older, the number of dead clusters increases. Therefore, only young cultures (2–4 days old) were used in all experiments.
10. In order to assess the intracellular labelling of Ac chemicals, Nile Red, a red fluorescent, live cell probe for LDs was used. All three Ac chemicals we found to colocalize completely with Nile Red, suggesting that Ac chemicals are specifically recognizing LDs in plant cells. Both blue and red emission range images were captured in single dye labeling experiments to assess the degree of channel crosstalk and autofluorescence. No signal from the Ac chemicals could be observed in the red detection range or Nile Red signal detected in the blue range. DMSO (1 %, v/v) did not induce any autofluorescence either in blue or red detection ranges.

11. Spectral imaging/lambda scan was used to further analyze comparatively the *in vivo* fluorescence emission characteristics of Ac chemicals, Nile Red and LD540. Peak intensity was observed at 460–480 nm detection interval for all the Ac chemicals. A wider emission curve extending into shorter blue wavelengths of the spectrum was observed for Ac-202, which displayed a more asymmetric emission curve with a pronounced shoulder at around 430 nm. Nile Red displayed emission across the middle of the visible spectrum peaking at 580–600 nm interval, overlapping the emission range of commonly used fluorescent reporters like YFP/dsRed. Although LD540 had a narrow emission range at 540 nm, it still overlaps the emission of YFP and partially GFP.
12. Ac chemical were tested on several monocot and dicot cultures at 10 μM for 15 min. Alfalfa (*M. sativa* ssp. varia A2) cultures showed high cytoplasmic background when labelled with Ac 201 and 202 but not 1041. But for maize (*Z. mays*, cv. H1233), tobacco (*N. tabacum* cv. Petit Havana SR1) and Arabidopsis (*A. thaliana* ecotype Landsberg erecta, MM1), Ac 1041 gave very weak signals. Maize culture labelled with Ac 201/202 displayed high LD heterogeneity. Multiple optical z-sections of labelled maize clusters were taken to prove the heterogeneity was not due to lack of dye penetration or single optical image acquisition. No emission crosstalk issues were encountered while imaging chlorophyll containing Arabidopsis cells labeled with Ac chemicals, blue LDs could be spectrally isolated from red chloroplasts with ease.
13. To test the potency of Ac dyes, we further tested them on germinating Arabidopsis seedlings. Both Ac-201 and 202 were equally suitable, labelling dense root meristem, fine root hairs, hypocotyl and epidermis of cotyledon. Owing to the bright fluorescence emission of Ac dyes, lower excitation energies were used to scan multiple times (~800 times) in 3D, Ac-202 labelled Arabidopsis cotyledon without significant dye bleaching. Epidermis of Arabidopsis cotyledon was found to contain significantly more LDs than the parenchyma region and this was not due to lack of dye penetration as we could observe LDs in parenchymal cells in the acquired serial optical z-sections.
14. To monitor LD variability in BY-2 (*Nicotiana tabacum* cultivar BY-2) cells, EdU labelled and fixed cells from every day samples (day 0-9) were co-labelled with 10 μM Ac-201 (labelling LDs), Alexa Fluor 488 azide (labelling incorporated EdU) and PI (to counterstain nuclei). Multiple confocal optical z-sections of cells with visible, well

defined cell borders were obtained. Identical imaging parameters were maintained throughout the image acquisition process over the course of the experiment. The LDs of individual cells were further analyzed quantitatively using the Huygens Professional image analysis software from Scientific Volume Imaging. Utilizing the Object Analyzer module of the Huygens Professional image analysis software, the total LD volume of individual cells (n=50) was calculated for each day, starting with day 0 and continued till day 9 (nine days after culture refreshment). Furthermore, to correlate the total LD content with cell cycle progression, the percentage of S-phase cells in all (0-9 days) samples were also calculated (n=500). We have observed that the total LD volume of the cells seem to be cycling through the different days of the growth curve of BY-2 culture. A striking discovery was the sudden drop of LD volume on day 2 in comparison to the high S-phase and low M-phase cells in that day. This could also be observed to a lesser degree on day 4.

15. To further investigate the correlation between total LD volume and S-phase of cell division cycle, BY-2 cells were blocked in S-phase with 5mM HU for a period of 36 hours and then the HU block was removed by successive conditioned media washes. Before wash and 2 hours after wash, BY-2 cells were labeled with Ac-201 and imaged. We have observed that the HU blocked cells had markedly less LDs in comparison to control cells, which showed abundant LD labeling. To confirm that this difference was not due to single optical section imaging, multiple z-optical section images were acquired. From these, it was apparent that there was a definite difference in the LD content of control and HU blocked cells. To affirm that the LD content difference seen between the samples was not due to cell death caused by HU block, the HU blocked and control cells were simultaneously labeled with PI. We did not find a marked difference between the PI labeling of control and HU blocked cells. With this, we could infer that the LD labeling difference between the samples was not caused by cell death due to HU block. 2 hours after HU block removal, control cells and HU block removed cells were also labeled with Ac-201 and imaged. We could observe a reemergence of LD in HU block removed cell. As with the before wash cells, multiple z-optical sections were obtained for both the control and HU block removed cells to confirm the reemergence of LDs.

Summary of findings and conclusions:

The main results of the work can be summarized as follows:

- Eleven novel fluorochromes were discovered labelling various, specific intracellular compartments using human HeLa cells as a model organism. Their labeling was confirmed by co-labeling each fluorochrome with a known marker. The fluorochromes were further studied for their optimum working concentrations. Each fluorochrome was assayed for its toxicity to assess its use for long term live cell experiments.

- Three of the fluorochromes (B2, B3 and C4) were found be to plant cell wall permeable and labelling LDs. Owing to their unavailability in large quantities other Ac chemicals derived from the same pool of in-house synthesized trifluoroaminophthalimide derivatives were used in our further studies. All three Ac chemicals tested (201, 202 and 1041) were plant cell wall permeable and confirmed to be labeling LDs by co-labeling with known LD marker Nile Red. The dyes were assessed for their toxicity and found to be non-toxic at concentrations up to 50 µM. Hence suitable for long term live cell analysis such as studying their mobility dynamics.

- Spectral emission characteristics of the Ac chemicals using lambda scan was studied. All three dyes with their narrow emission range, displayed a peak intensity at 460-480 nm, well isolated from green and red colored dyes and fluorescent proteins, providing the much needed flexibility for multi-color imaging.

- Ac chemicals were tested on several monocot and dicot plant cultures; Ac 201/202 displayed high background in alfalfa cultures whereas Ac 1041 showed discrete LD labelling. The staining intensity and/or penetration of Ac 1041 was less favorable in other plant cultures. Spectral emission and labelling intensity differences between the highly similar Ac chemicals suggest that, additional modifications might yield even better LD probes with superior characteristics.

- Ac chemicals were also tested for their labelling properties on intact green tissues. Ac-201 and 202 were found to penetrate deep multilayered tissues of intact seedlings. Both underground and aboveground tissues of seedlings showed several LDs stained with Ac

chemicals. Repetitive laser scanning during collection of multiple z-axis optical sections on stained cotyledons did not cause significant signal fading of Ac chemicals. It was found that the epidermal cells of Arabidopsis cotyledons have more LDs as compared to parenchyma cells.

- Employing the novel LD dyes, the LD variability in BY-2 cells across their growth curve was studied. It was observed that the total LD volume of the cells were cycling through the different days of the growth curve of BY-2 culture. Furthermore, we could observe a sudden drop of LD volume on day 2 in comparison to the high S-phase cells in that day.

- BY-2 cells blocked in S-phase using 5mM HU had a considerable decrease in LD as compared to control cells. Two hours after HU block removal, we could observe a reemergence of LDs.

Publications

The Ph.D. thesis is based on:

1. Soujanya Kuntam, László G. Puskás, Ferhan Ayaydin (2015) Characterization of a new class of blue-fluorescent lipid droplet markers for live-cell imaging in plants. Plant Cell Rep, 34:655-665. IF: 3.071
2. Soujanya Kuntam and Ferhan Ayaydin (2015) Detection of S-phase of cell division cycle in plant cells and tissues by using 5-ethynyl-2′-deoxyuridine (EdU). In: Plant Microtechniques and Protocols, Springer International Publishing, pp 311-322.
3. Eszter Molnár*, Soujanya Kuntam*, Pradeep Kumar Reddy Cingaram, Begüm Peksel, Bhavyashree Suresh, Gabriella Fábián, Liliána Z. Fehér, Attila Bokros, Ágnes Medgyesi, Ferhan Ayaydin, László G. Puskás (2013) Combination of small molecule microarray and confocal microscopy techniques for live cell staining fluorescent dye discovery. Molecules, 18:9999-10013. IF: 2.416

(* EM and SK contributed equally to this work)