# Laboratory Exercise

# Using Fluorescent Lipids Contributes to the Active Learning of Principles Underlying Lipid Signaling

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## Abstract

The concepts of phospholipase activity is often taught in undergraduate biology and biochemistry classes and reinforced in laboratory exercises. However, very rarely does the design of these exercises allow students to directly gain experience in the use of modern instruments such as digital imaging systems and fluorescence spectrophotometers. The laboratory exercise described here involves the use of fluorescent lipids to evaluate phospholipase activity. Students use thin layer chromatography (TLC) to understand how lipids change under different conditions (i.e. abiotic and biotic stress). They explore strategies to separate, visualize and quantify lipids by TLC, digital imaging, and fluorometry. They also have increased opportunities for hands-on practise with experimental design, liposome sample preparation, and implementation of instrumentation commonly used by experienced researchers; all while learning and applying fundamental concepts about lipids. © 2018 International Union of Biochemistry and Molecular Biology, 47(1):100–105, 2019.

***Keywords:*** *Fluorescent lipids; thin layer chromatography; phospholipase D; phospholipase A*

## Introduction

Phospholipases are a ubiquitous group of enzymes that catalyze the hydrolysis of acyl esters and phosphate esters on phospholipids (diacylglycerophosphate esters and related compounds). Phospholipases are also defined by the position they hydrolyze on the phospholipid. These enzymes can influence stress tolerance through alterations in the composition of plasma membrane lipids. Several phospholipid-based signaling pathways are rapidly activated in response to abiotic and biotic stress [1]. For example, the phospholipase A (PLA) hydrolyses the carboxylic esters at the sn-1 (PLA1) or sn-2 (PLA2) positions of glycerol backbones. Phospholipase D (PLD) is a phosphodiesterase that catalyzes the hydrolysis of a head group, leaving to yield phosphatidic acid (PA) and a head group. A classical method to specifically measure PLD activity *in vivo* is based on its ability to transfer the phosphatidyl moiety of a structural lipid to a primary alcohol (Fig. 1). Conventional laboratory practices make use of $^{32}P$ prelabelled structural lipids [2]. Enzyme assays aimed at understanding lipid signaling are one of the main topics addressed in undergraduate biochemistry laboratory courses. They often involve monitoring the appearance or disappearance of either the lipid substrate or the product with TLC method [3]. One common method is to label the cell/tissue with $^{32}P$ and monitor the phosphotransfer from $^{32}P$-□γ-ATP to the respective substrate, and then measure the enzymatic activity [4]. The advantage of this method is that we can test any nonradioactive phosphate acceptor (lipid), since the subsequent reaction with γ-$^{32}P$-ATP gives a radioactive product. A disadvantage is that separating the resulting monophosphate and unreacted ATP is quite a tedious procedure. The need to work with high-energy emissions of $^{32}P$ is another drawback. Moreover, when working with phosphorus a fresh preparation must be purchased each time because of its short half-life. All of these downsides have made it necessary to design improved phospholipase activity assays. We looked into the use of a fluorescent probe as substrate of phospholipase. Phospholipids labeled with the 7-nitrobenz-2- oxa-1,-3-diazol-4-yl (NBD) fluorophore in one of the acyl chains are commercially available for all major phospholipid classes. NBD-lipids have been used extensively as fluorescent analogues of native lipids in biological and model membranes to study a variety of processes. The goal was to use a

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fluorescent lipid analogue, a fatty acid labeled "NBD-phosphatidylcholine"(NBD-PC), as a substrate for PLD and PLA2 assays. A simple experimental system was designed, taking into consideration that chilling stress triggers PLD activity and that PLA2 is one of the main responses to biotic stress. The laboratory exercise presented here is a 3-week experiment intended to give undergraduate students hands-on experience with enzymatic reactions, lipid extraction and separation, digital imaging systems, the use of spectrofluorometers, and the corresponding data interpretation. In the first part of this experiment, students are asked to work with crude protein in order to challenge them to determine the amount of protein necessary for the phospholipase assay. Then, they prepare the liposome (spherical vesicle having at least one lipid bilayer) and start up the enzymatic reaction. Later, they extract and separate the lipids by TLC. In the second part, they expose the TLC with Digital Imaging System, to capture the image and identify the spots. Eventually, they scratch out the spots of TLC to elute the lipid from the silica and quantify fluorescence by fluorometry. By the end of this exercise, students are expected to have a better understanding of lipid signaling. In that respect, the exercise was designed with the concept of active learning in mind [5]. In particular, students must engage in higher-order thinking tasks such as analysis, synthesis, and evaluation. With active learning students' engagement operates on two levels: they both do things and think about the things they are doing. Here, we describe the design of this new teaching methodology and the results obtained in the first two academic years after its implementation. Our data reveal improvement on student performance, which suggests that this methodology results in corresponding learning gains.

## Experimental Procedures

### Plant Materials, Growth Conditions, and Stress Treatment

Barley (*Hordeum vulgare*, scarlett) seeds were germinated at 25 °C for 4 days (control). For abiotic stress: short-term chilling was induced by incubating seedlings at 4 °C for different times (30, 60, or 180 min) [6]. For biotic stress, to obtain *Fusarium graminearum* macroconidia, an active culture of the fungus grown on solid Spezielier Náhrstoffarmer (SNA) medium (g/l: 1 $KH_2PO_4$, 1 $KNO_3$, 0.5 $MgSO_4$-$7H_2O$, 0.5 KCl, 0.2 glucose, 0.2 sucrose, 20 Agar, at pH 5.4), was transferred by cutting the medium into small agar pieces that were placed in liquid SNA medium at 28 °C, with 150 rpm shaking. After 7 days, the culture was filtered through a 0.5 mm polyester filter. Then, macroconidia were harvested by centrifugation (11,000 × g, 15 min) and resuspended in a minimal volume of sterile glycerol 15%. To infect the plant root system, a solution of macroconidia was prepared at a concentration of $1 \times 10^7$ conidia/ml in an improved Neubauer chamber. Finally, the seedlings were put in a sterile petri plate with the macroconidia solution in contact with the roots. For control samples, water was used and the treatment was alternatively stopped and started for 30 sec intervals.

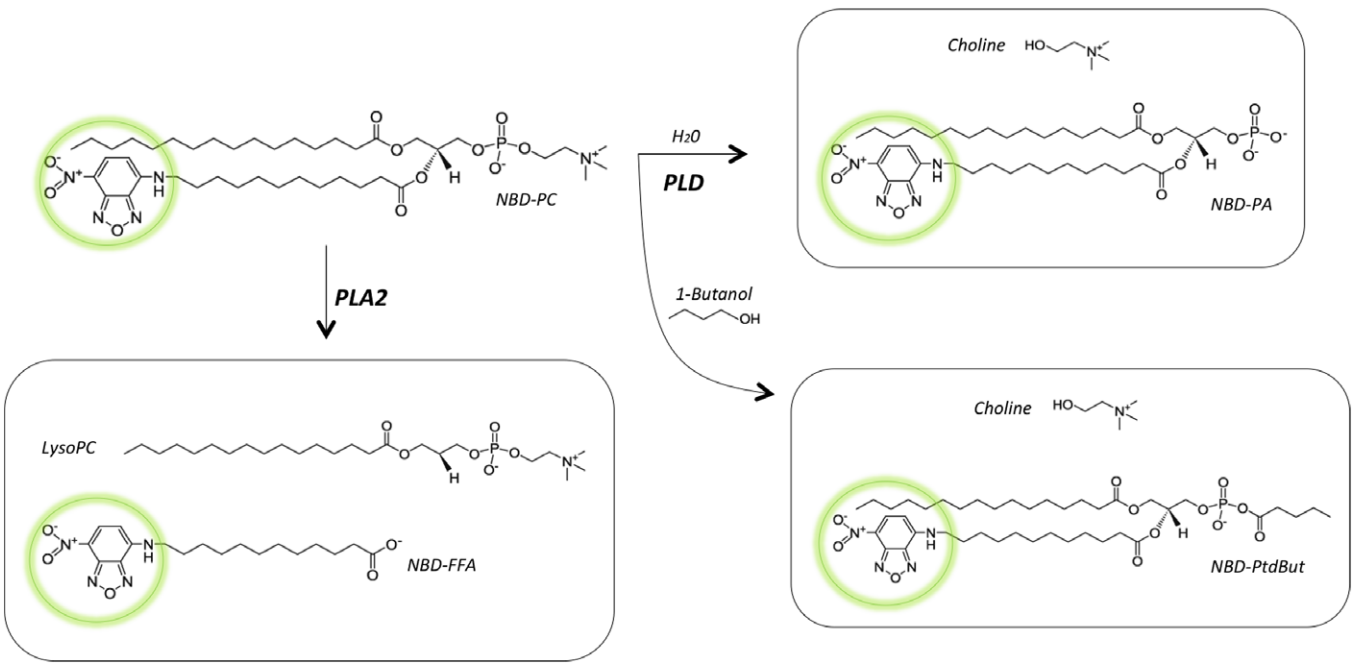


**FIG 1** *Schematic representation of PA and PtdBut formation by PLD-mediated transphosphatidylation activity and lyso-PC and FFA formation by PLA2 with NBD-PC substrate. [Color figure can be viewed at wileyonlinelibrary.com]*

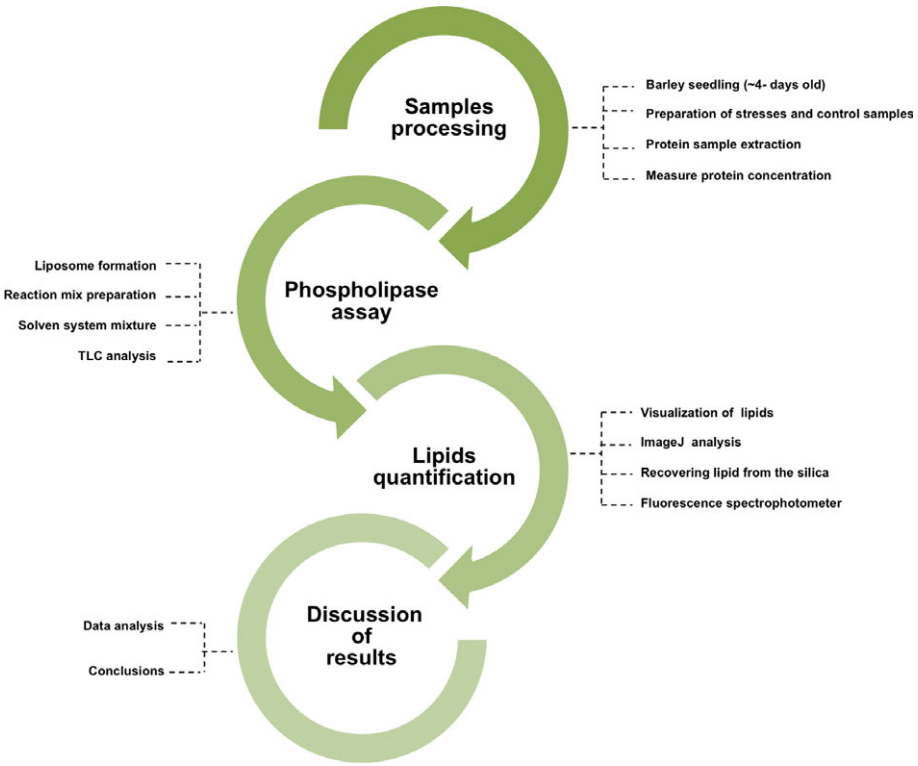


**FIG 2** *Laboratory exercise workflow. [Color figure can be viewed at wileyonlinelibrary.com]*

## Phospholipase Enzymes Assay

### Protein Extraction

Total proteins from roots were extracted as described previously Meringer, *et al*. [7]. In brief, 500 mg tissue was ground by mortar with 400 μL homogenization buffer (50 mM Hepes, 10 mM KCl, 1 mM EDTA, 0.5 mM sucrose, pH 7.5) and 20 μL protease inhibitors (1 mg $mL^{-1}$ leupeptin, 1 mM phenyl methane sulfonyl fluoride (PMSF), 1 mg $mL^{-1}$ aprotinin) at 4 °C. The homogenate was centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was collected, and protein content was determined following the Bradford method [8]. PLD and PLA2 activity were assayed directly from the supernatant.

### PLD Activity Assay

PLD activity was determined by TLC as the synthesis of phosphatidylbutanol (NBD-PtdBut) in relation to NBD-PA and NBD-PC levels [6]. NBD-PC, (Avanti Polar Lipids, Inc—Alabama-USA) was stored at −80 °C in chloroform (1 mg $mL^{-1}$), dried under an $N_2$ stream before its use, resuspended in Hepes (50 mM, pH 7.4), and added to the PLD assay mixture as a liposome. The standard PLD assay mixture consisted of 20 mM Mes-NaOH (pH 6.5), 50 mM $CaCl_2$, 0.25 mM SDS, 1.5 μL fluorescent substrate (NBD-PC, 10–50 μg), 1% (v/v) 1-butanol, and 40 μg proteins, in a total volume of 40 μL. The reaction was initiated by addition of the substrate. Incubation continued for 30 min at 30 °C with shaking (100 rpm), and the reaction was stopped by addition of 150 μL chloroform/methanol (1:2, v/v), 40 μL chloroform, and 40 μL 2 M KCl. The mixture was centrifuged at 15,000 × g for 2 min, and the phases were separated. The aqueous phase was added with 100 μL chloroform and centrifuged at 15,000 × g for 2 min. The lower chloroform phases from each step were pooled. Samples were dried under an $N_2$ stream, resuspended in a minimal volume of chloroform/methanol (95:5, v/v), spotted on TLC plates (silica gel G; Fisher Scientific), and developed with 2,2,4-trimethylpentane/acetic acid/$H_2O$/ethyl acetate (2:3:10:13, v/v). Fluorescence from lipids (excitation wavelength 460 nm, emission wavelength 534 nm) was measured using a fluorescence spectrophotometer (Image Station 4000 MM PRO-Carestream, Molecular Imaging) and quantified with the ImageJ software program. PLD activity was determined by formation of NBD-PtdBut. NBD-PtdBut was expressed as the percentage of NBD-PtdBut fluorescence, normalized to NBD-PC, and expressed as fold increase relative to time 0 value (data not shown).

### PLA Activity Assay

Liposomes were prepared by drying a mix made up of 186.7 μg soy PC (Sigma P3644) and 13.3 μg fluorescently labeled phospholipid in 100 μL chloroform under a stream of nitrogen, followed by resuspension in 50 mM Tris, pH 8.0, 100 mM KCl, and 0.1% (w/v) sodium deoxycholate at

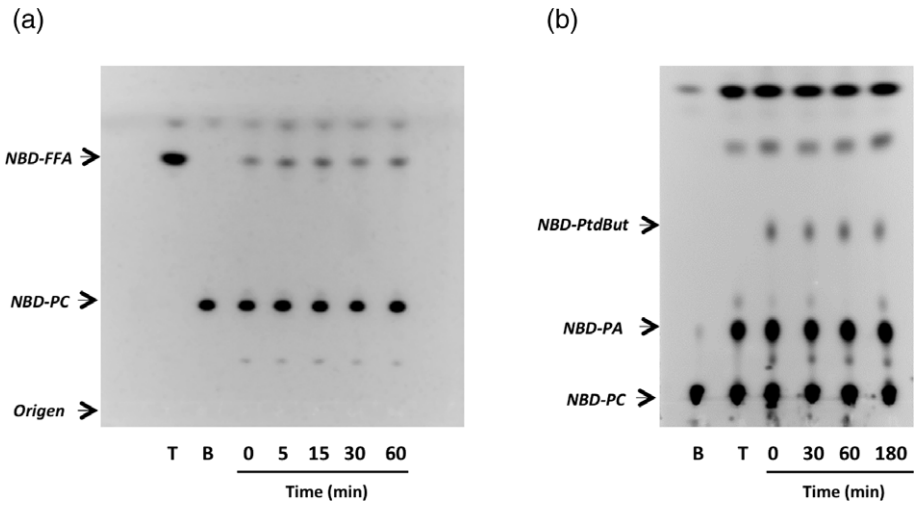


**FIG 3**

*Effect of biotic and abiotic stress on phospholipase activity. (a) Image of a TLC plate showing NBD-FFA formation in barley roots stimulated with* Fusarium graminearum. *(b) Representative TLC plate of PLD activity, with lipids separated with the solvent containing ethyl acetate. B: control without proteins; T: control without 1-butanol; 0: control roots; 30, 60, 180: time course NBD-PtdBut formation in chilling response. The locations of NBD-FFA, NBD-PC, NBD-PtdBut, NBD-PA are indicated by arrows.*

a concentration of 1 μg $\mu L^{-1}$ and sonication for 5 min to achieve a clear suspension. For enzymatic tests, protein (100 μg) was incubated with 3 μL of liposomes in a total volume of 100 μL of MES-KOH, pH 6.8, and 1 mM $CaCl_2$ at 33 °C for 30 min. Reactions were stopped by adding two volumes of the stopping solution (methanol:chloroform 2:1, v/v), 1 volume of 0.1 m KCl was subsequently added, and incubation at −20 °C for 20 min followed. After centrifugation for 30 sec at 10,000 × g, the organic phase was evaporated under a stream of nitrogen and redissolved in 10 μL chloroform for TLC on silica gel 60 (Merck, Darmstadt, Germany) in a solvent of chloroform:methanol:$H_2O$ (65:25:4, v/v). Plates were dried and scanned. Fluorescence (excitation 460 nm, emission 534 nm) from lipids was measured with a fluorescence spectrophotometer (Image Station 4000 MM PRO-Carestream Molecular Imaging) and quantified with ImageJ.

### Quantification of Fluorescent Lipids

Fluorescently labeled lipids were visualized with a UV light box (FBTIV-88, Fisher Scientific), and the regions corresponding to NBD(-PC, -PA, -PtdBut, -FFA) were marked. The spots marked were scraped from the plates and placed in 600 μL chloroform:methanol:$H_2O$ (5:5:1, v/v/v), vortexed, and centrifuged for 5 min at 15,000 x g. Fluorescence (excitation 460 nm, emission 534 nm) from the eluted lipids was measured with a fluorescence spectrofluorometer (Horiba-Fluoromax-3) [9]. Lipid spots were identified by comparison to standards (Avanti Polar Lipids, Inc—Alabama-USA) and visualized with iodine vapor. The difference in relative mobilities between NBD-labeled and unlabelled standards was less than 5%. A standard curve was constructed using a range of phosphatidylcholine (PC) concentrations. The relationship between the amount of PC standard and the fluorescence measured was linear throughout the range 6–50 ng. These values could be used to express the results as enzymatic activity in μKat (microkatal).

### Safety Considerations

Some species of *Fusarium graminearum* produce mycotoxins, so gloves must be worn at all times. Organic solvents should be used under a fume hood. Our institution has compiled its own Laboratory Advisory Guidelines that provide procedural information for laboratory workers about how to dispose of laboratory waste. These guidelines should be read in conjunction with Hazardous Waste Disposal Guidelines, in order to minimize risks.

### Timing and Student Participation

Before the first laboratory day, students were required to read a worksheet that introduced the topics and activities to be covered. The instructor was responsible for preparing the stresses and the control barley seedlings in advance. The students prepared the remaining samples and reagents. They worked in pairs throughout the exercise and typically finished in approximately 4 hr, with the exception of the third laboratory day, when they operated the spectrofluorometer.

## Results

The National University of Rio Cuarto, a primarily undergraduate institution, has a Horiba-Fluoromax-3 fluorescence spectrophotometer and a Carestream Image Station 4000MMPRO. These instruments are used to support undergraduate laboratory courses and research. This laboratory exercise has been performed in our biochemical laboratory techniques course for 3 years in a row, always in the first

4 months, with slight modifications each year. The final version presented here has been performed two times in the signaling transduction laboratory course, which is required for microbiology and biology students. In order to be allowed to take part in laboratory exercises, it is a prerequisite for students to have taken 4 months of organic chemistry, quantitative chemical analysis, biochemistry I, and introduction to biochemical laboratory techniques. A maximum of six students can enroll in the Signaling Transduction Laboratory course and there are weekly 4-hr meetings during 8 weeks. This procedure was designed to be covered over the course of three practical 4-hr classes, that is, a total of 12 hr of work for the students. This included time for students to complete the exercise and for the instructor to facilitate in-class discussions. In a theoretical class, students were given an explanation on the experimental system and the workflow. On the first laboratory day, students became familiar with the preparation of samples from plant root tissues, the isolation of protein membranes, and the determination of protein concentration by Bradford methods. On the second day, they reviewed the procedures to determinate phospholipase activity and prepared NBD-PC substrate as liposome. The instructor explained how liposomes are formed, as follows. Liposomes or lipid vesicles result from the hydration of thin lipid films or lipid cakes where stacks of liquid crystalline bilayers become fluid and swollen. When agitated, the hydrated lipids sheets detach close themselves to form large, multilamellar vesicles that prevent water from interacting with the hydrocarbon core of the bilayer at the edges. Once these particles have formed, sonic energy (sonication) or mechanical energy (extrusion) are necessary to reduce their size. Sonication was the chosen method for this laboratory exercise. For the phospholipase assay, a Master Mix can be prepared for multiple reactions. Preparation of this Master Mix eliminates the need to repeatedly pipette volumes, so the samples are more consistent with each other. Some basic procedural recommendations should be heeded to ease the progression of the exercise. The reaction is initiated by adding NBD-PC substrate and then stopped by adding the solvent mix. It is advisable to equilibrate the tip of pipet with chloroform by pipetting it up-and down for a number of times, and use cold solvents since they pipet better. Before loading for TLC, a line should be drawn with soft pencil, 2 cm from the bottom, where the samples are to be spotted. The samples are also marked with a small dot, 0.5–1.5 cm apart. TLC is started by placing the plate into the solvent and then run for 0.5 hr. The plate is taken out and left to dry in the fume hood for 1 h to eliminate solvent fumes which would affect the phosphorimager screen. The TLC is exposed for ~1 min, though more time should be contemplated for higher resolution images. A typical TLC pattern of phospholipase activities is shown in Fig. 3. The rapid response of PLA2 activity evoked by *Fusarium graminearun* can be seen in Fig. 3*A*. Phospholipase activity increased rapidly and transiently at 5 min and then it showed values similar to those of the control treatment. *Naja naja* venom PLA2 from Sigma-Aldrich was used to generate *lyso*-PC as a lipid reference. Chilling modulated PLD activity, increasing NBD-PtdBut formation at 180 min (Fig. 3*B*). It is important to mention that the two-phospholipid patterns obtained can be generated by phospholipase action using NBD-PC. While TLC was running, the instructor showed the students the phosphorimager, described the instrumental components, and had students practice with the help of an archive image. The instructor then handed out information about the samples used for this experiment, led a discussion on lipid signaling modulated by stress, and asked the students to sketch a theoretical TLC of expected results. Most students drew a TLC that included several lipids, with each spot corresponding to a product of the NBD-PC hydrolysis. This subsequently led to a discussion about the differences in phospholipase activity during either biotic or abiotic stress. Approximately half the students had identical results for all samples in their

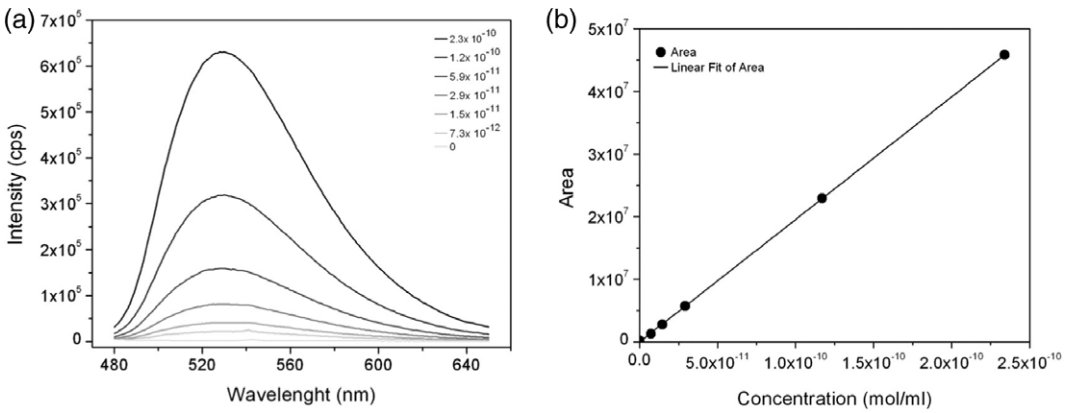


**FIG 4** *Fluorescence emission spectrum from irradiated solutions of NBD-PC at different concentrations between 480 and 650 nm (a). Calibration plot to determine the lipid content in unknown samples: integrated area of peaks from fluorescence emission spectrum plotted against the content of fluorescent lipid (b).*

predicted TLC. The instructor referred students to the enzymatic activity and asked them to consider how they would calculate it. To do that, fluorescently labeled lipids (were visualized with a UV light box) and the regions corresponding to NBD(-PC, -PA, -PtdBut, -FFA) were marked by the students. The spots marked were scraped from the plates and fluorescence from the eluted lipids measured with a spectrofluorometer. Peaks observed were analyzed to calculate the area under the curve using Origin 8 (Fig. 4*A*). Finally, these data were converted to concentration by means of a calibration plot (Fig. 4*B*), divided per unit of time and converted to μKat.

## Conclusion

For the successful implementation of this experiment in a typical 4-hour biochemistry laboratory period, several factors should be considered. First, given the limitations of the methods, groups of no more than three or four students are able to perform this experiment in the time allotted. Secondly, there were minor differences (less than 5%) between groups in the calculations for enzymatic activity. This discrepancy was mainly due to the difference in the marked spot area as well as potential pipetting differences. When all student groups used the same stock solutions (i.e. master mix, liposome samples, etc.), results were more consistent. Finally, it should be stressed upon students that the main goal of this experiment is to demonstrate the formation of NBD-products over time using NBD-PC, and if quantitative results were required, additional measurements would be needed. In summary, this experiment allows students to directly assay enzymatic activity through the use of NBD-PC and highlights the use of an "alternative fluorescently labelled substrate" in enzymatic experiments. Phospholipases, the specific enzymes studied, are an excellent choice to understand lipid signaling mainly due to the clear separation of product signal. However, one could imagine other interesting systems worthy of exploration in the undergraduate biochemistry laboratory.

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