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Protein–Lipid Interactions

# In Vivo Profiling and Visualization of Cellular Protein–Lipid Interactions Using Bifunctional Fatty Acids**

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Cellular processes are mediated by the concerted action of numerous biomolecules that form complex interaction networks. Considerable efforts have been devoted to elucidating the cellular interactome, with the majority of studies focusing on mapping protein–protein, protein–DNA, and protein–metabolite interaction networks.[1,2] Yet two-thirds of the cellular proteome operates at a membrane surface or within a membrane comprising thousands of different lipid species. Besides serving as essential building blocks of membranes and anhydrous stores of energy, lipids participate in a multitude of signaling pathways. Perturbations in lipid homeostasis frequently result in human diseases, ranging from neurodegenerative disorders to metabolic syndrome and cancer.[3,4] While these findings imply an intricate interplay between proteins and lipids, only a few studies have been carried out to chart protein–lipid interactions in a systematic fashion.

To catalogue protein–lipid interactions in yeast, Gallego et al. used a comprehensive set of tandem affinity-purified proteins and probed these on miniaturized nitrocellulose lipid arrays; this work led to the novel concept that pleckstrin homology domains can have dual binding specificities and may act as coincidence sensors for distinct lipid classes.[5] Other studies focused on the application of radioactive photoactivatable lipids to investigate individual protein–lipid interactions in living cells.[6,7] This approach recently led to the identification of a preferential interaction of the membrane protein p24 with a single species of sphingomyelin, with the lipid serving as a cofactor in the formation of coat protein I (COPI) vesicles.[8] In addition, phospholipid-based activity probes have been applied to characterize lipid-binding proteins using synthetic analogues of phosphatidylcholine and phosphatidylinositol polyphosphates.[9–11] Apart from a photoaffinity moiety for the covalent labeling of target proteins, these probes contain a second tag for click chemistry to enable the detection and enrichment of the crosslinked proteins for subsequent identification by mass spectrometry. However, a major disadvantage of these bifunctional phospholipid analogues is that their application is restricted to cell extracts, thus preventing a systematic analysis of protein–lipid interactions in their native environment.

For a global analysis of protein–lipid interactions in living cells, we have now synthesized a 15 carbon long photoactivatable and clickable fatty acid, pacFA (Figure 1a). In the initial step, the diprotected keto fatty acid **3** was obtained from the Grignard reaction of the trimethylsilyl(TMS)-protected alkyne **1** and acid chloride **2**. In the second step, the ester and TMS group were cleaved simultaneously under alkaline conditions yielding the keto fatty acid **4**. Conversion of the keto group into a photoactivatable diazirine ring was achieved as described previously,[6] yielding pacFA **5**.

To screen for protein–lipid interactions in vivo, cells were fed pacFA as a precursor for the biosynthesis of bifunctional lipids. Proteins in direct contact with a bifunctional lipid were then crosslinked by irradiation of the diazirine group with UV light (Figure 1b). This resulted in a highly reactive intermediate that, within the nanosecond range,[12] formed a covalent linkage to molecules in close proximity. Next, click chemistry was used to label the alkyne group with a reporter

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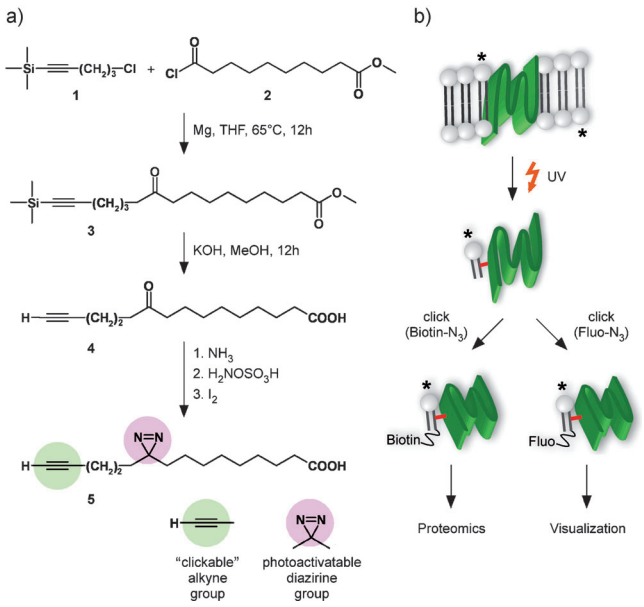


***Figure 1.*** a) Synthesis of the bifunctional fatty acid pacFA **5**. b) General scheme for the in vivo profiling of cellular protein–lipid interactions. pacFA fed to cells is metabolically incorporated into a bifunctional lipid (marked by asterisk), which can be crosslinked to its protein-binding partner (green) by UV irradiation. Click chemistry is used to label the alkyne group in the bifunctional lipid with biotin-$N_3$ or fluorescein-$N_3$ (Fluo-$N_3$), allowing the respective identification or visualization of the crosslinked protein–lipid complex.

molecule, allowing the visualization or affinity-purification of the crosslinked products. An important advantage of this two-step procedure is that the functionalized lipid is tagged after its interaction with a protein-binding partner has been captured by UV-crosslinking, so that the bulky reporter molecule does not interfere with the protein–lipid interaction.

As a proof-of-principle experiment, pacFA was incubated with the fatty acid carrier protein bovine serum albumin (BSA) and subjected to UV irradiation. Next, the mixture was subjected to a click reaction with biotin azide and analyzed by immunoblotting. Tagging of BSA with biotin was observed only after UV irradiation and in the presence of pacFA (Figure S1 in the Supporting Information). Biotinylation of BSA was strictly dependent on $CuSO_4$ and the reducing agent tris(2-carboxyethyl)phosphine (TCEP), two essential components of the click reaction. No photoaffinity labeling was observed when BSA was replaced with lysozyme, a protein lacking a known fatty acid binding site. Thus, photoaffinity labeling of BSA relies on a specific interaction between the protein and pacFA.

To investigate whether pacFA is a suitable precursor for the biosynthesis of phospholipids, we fed pacFA to Chinese hamster ovary (CHO) and human HeLa cells for 16 h and analyzed cellular lipid extracts for the presence of bifunctional species of phosphatidylcholine (PC) using nano-electrospray ionization tandem mass spectrometry (nano-ESI-MS/MS). The labeling of CHO cells with pacFA yielded peaks that showed a 8 Da increase in the $m/z$ ratio relative to the peaks of endogenous PC species, a shift corresponding to the difference in the molecular weights of pacFA (264 g mol$^{-1}$) and its natural analogue, palmitic acid (256 g mol$^{-1}$; Figure S2 in the Supporting Information). Multiple precursor ion scans (MPIS) of lipid extracts from pacFA-fed human HeLa cells for the characteristic pacFA ion ($[M-H]^-$, $m/z$ 263) revealed the presence of various pacFA-containing PC species (Figure S3a in the Supporting Information). pacFA was also incorporated into phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), and cardiolipin (CL) (Figures S3b and S4 in the Supporting Information).

To monitor the metabolic turnover of pacFA, lipid extracts of pacFA-fed HeLa cells were subjected to a click reaction with 3-azido-7-hydroxycoumarin and then analyzed by thin-layer chromatography as described for other alkynylated lipids.[13] Within 1 h of metabolic labeling, the bulk of pacFA (> 95 %) was incorporated into diacylglycerol (DAG, 16 %), triacylglycerol/cholesterol ester (TAG/CE, 17 %), and phospholipids (49 %; Figure S5 in the Supporting Information). Increasing the labeling time to 16 h or substituting pacFA for palmitic acid alkyne, a clickable fatty acid (cFA), resulted in a very similar distribution of the label over these major lipid classes. Thus, pacFA is readily converted into a variety of bifunctional lipids including all major classes of phospholipids. In contrast, we found no evidence for the incorporation of pacFA into sphingolipids, a somewhat surprising result as palmitic acid is a precursor for the production of sphinganine and ceramide by serine palmitoyltransferase and ceramide synthase, respectively. As both enzymes display a high level of specificity towards acyl chain length,[14–16] the introduction of the diarizine and alkyne groups may cause a length mismatch that prevents the recognition of pacFA as a substrate for sphingolipid biosynthesis.

We next investigated the application of pacFA as a tool for capturing protein–lipid interactions in living cells. To this end, CHO cells were fed pacFA for 1 h or 16 h followed by UV irradiation and cell lysis. The cell membranes were separated from the cytosol and subjected to a click reaction with Alexa 488 azide. Alexa-labeled proteins were visualized by SDS-PAGE and in-gel fluorescence. As shown in Figure S6 in the Supporting Information, a subset of membrane proteins in pacFA-fed, UV-irradiated cells was fluorescence-labeled whereas cytosolic proteins were essentially devoid of labeling. In the absence of UV irradiation, a subset of membrane proteins was labeled, but only in cells fed with pacFA for 16 h (Figure 2a). No labeling was observed when pacFA was omitted. Thus, in cells fed with pacFA for 16 h, some membrane proteins undergo fluorescence labeling irrespective of photo-crosslinking. As a small fraction of cellular proteins is modified by palmitoylation, the labeling of proteins in non-UV-irradiated cells could be due to the metabolic incorporation of pacFA. Consistent with this notion, UV-independent labeling of proteins was also observed in cFA-fed cells (Figure S7 in the Supporting Information).[17] To address this further, total membranes from pacFA-fed non-UV-irradiated CHO cells were subjected to a click reaction with biotin azide. Click reactions on membranes from cells grown in the absence of pacFA served as a control. Next, biotin-derivatized proteins were visualized by immunoblotting and affinity-purified using Neutravidin

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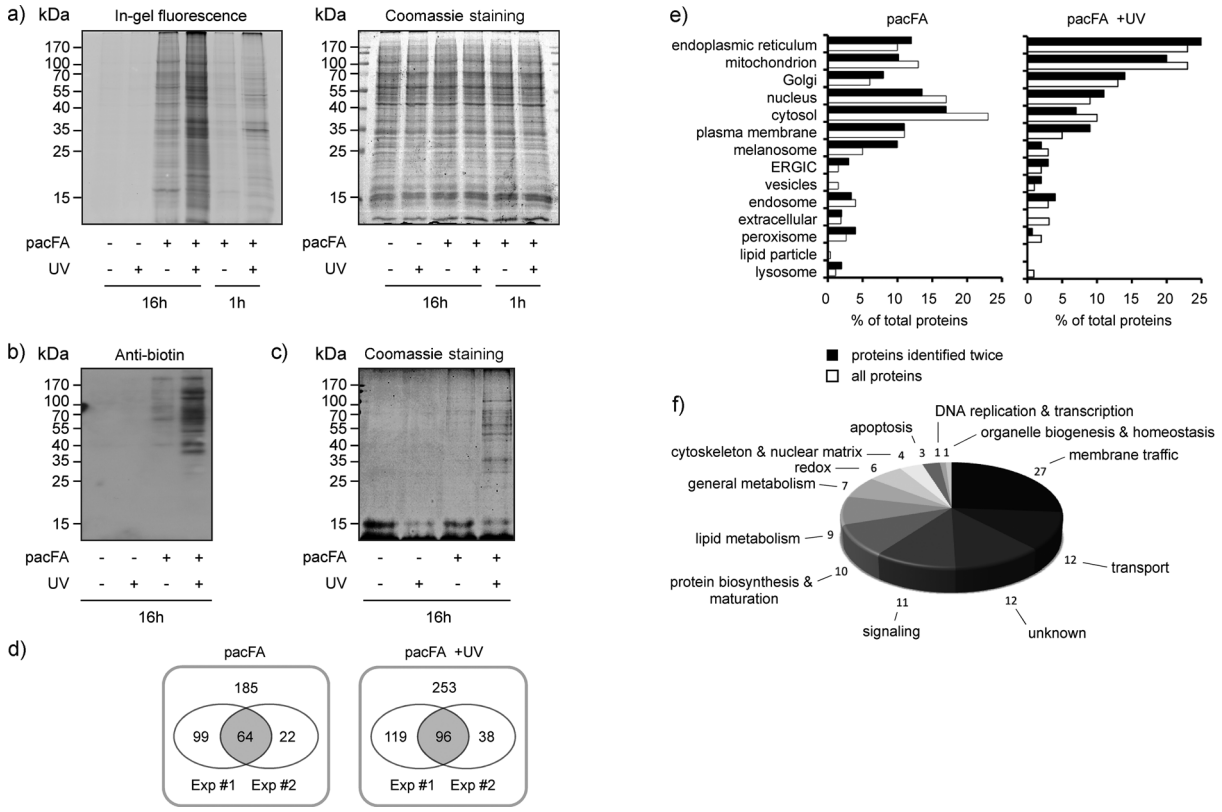


***Figure 2.*** Global profiling of protein–lipid interactions in pacFA-fed CHO cells. a) CHO cells were grown for 1 h or 16 h in the presence or absence of pacFA and then subjected to UV irradiation, as indicated. Total cellular membranes were isolated, subjected to a click reaction using Alexa 488 azide, processed for SDS-PAGE, and then analyzed by in-gel fluorescence (left panel) or stained with Coomassie brilliant blue (right panel). b) Total membranes from CHO cells treated as in (a) were subjected to a click reaction using biotin azide, processed for SDS-PAGE, and then subjected to Western blotting using an anti-biotin antibody. c) Biotinylated protein–lipid complexes were purified from total cellular membranes using Neutravidin beads and then subjected to SDS-PAGE and staining with Coomassie brillant blue. Note that the prominent bands at approximately 15 kDa correspond to monomeric subunits of Neutravidin. d) Mass spectrometric identification of proteins affinity-purified with Neutravidin from pacFA-fed CHO cells prior to (left panel) or after UV irradiation (right panel). Presented are the numbers of proteins that were identified from two independent experiments. e) Subcellular distribution of the proteins identified in (d). f) Clustering of functions assigned to proteins affinity-purified from pacFA-fed, UV-irradiated CHO cells that were identified twice in two independent experiments.

beads (Figure 2b,c). The purified proteins were separated by SDS-PAGE, subjected to in-gel tryptic digestion and identified by mass spectrometry (criteria for the identification of crosslinked proteins are defined in the Supporting Information). Two independent experiments yielded a total of 185 proteins that were exclusively present in affinity-purified fractions of pacFA-fed non-UV-irradiated cells, of which 64 proteins were identified twice (Figure 2d, Table S2 in the Supporting Information). About 6.5% of these proteins (12 out of 185) are known targets of palmitoylation, whereas 26% (48 out of 185) correspond to proteins identified previously in a global screen for palmitoylated proteins.[18] Moreover, about half of the 185 proteins are predicted to localize in compartments where most palmitolyated proteins are known to occur (i.e. cytosol, nucleus, plasma membrane; Figure 2e).[19] Together, these results indicate that the affinity-purified proteins of non-UV-irradiated pacFA-fed cells are enriched in palmitoylated proteins and that pacFA is incorporated into palmitoylated proteins.

To identify proteins that are crosslinked to bifunctional lipids, the total membranes from pacFA-fed UV-irradiated CHO cells were reacted with biotin azide and the proteins were affinity-purified using Neutravidin beads prior to mass spectrometric analysis. Non-UV-irradiated pacFA-fed cells served as a control to distinguish lipid-crosslinked proteins from palmitoylated proteins. Two independent experiments yielded a total of 253 proteins that were either exclusively present or highly enriched in affinity-purified fractions of pacFA-fed UV-irradiated cells. Nearly 40% of these lipid-crosslinked proteins (96 out of 253) were identified twice (Figure 2d; Table S3 in the Supporting Information). In contrast to the proteins that were affinity-purified from non-UV-irradiated cells, the bulk of the lipid-crosslinked proteins isolated from UV-irradiated cells corresponded to endoplasmic reticulum (ER) resident or mitochondrial proteins (Figure 2e). These proteins participate in a wide range of cellular processes, including lipid metabolism, protein biosynthesis, membrane trafficking, transport, and signaling (Figure 2f).

To further validate our findings, we identified lipid-crosslinked proteins from pacFA-fed, UV-irradiated HeLa cells (Figure S8 in the Supporting Information). In one

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experiment, HeLa cells were fed pacFA for 16 h prior to UV irradiation. In a second experiment, the pacFA labeling time was shortened to 1 h. Omission of UV irradiation served as a control to discriminate lipid-crosslinked proteins from palmitoylated proteins, as described above. As shown in Figure 3a, the number of putative palmitoylated proteins

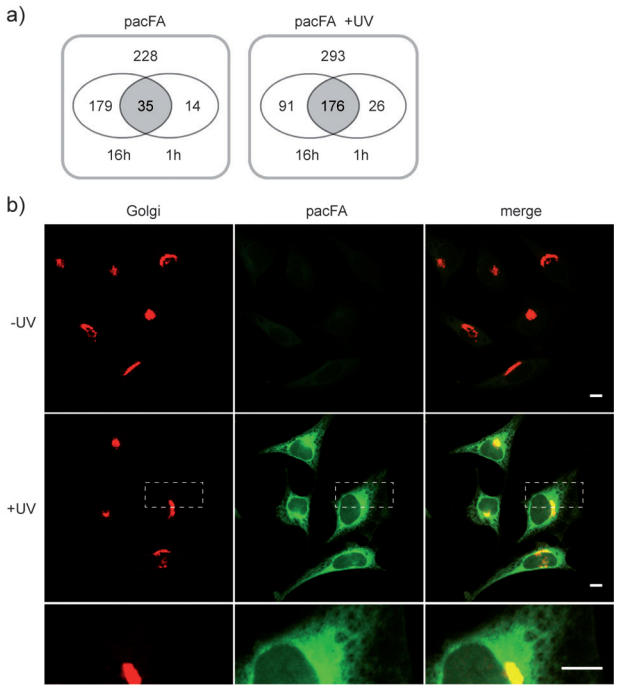


***Figure 3.*** Imaging of protein–lipid interactions in pacFA-fed HeLa cells. a) Mass spectrometric identification of proteins affinity-purified with Neutravidin from pacFA-fed HeLa cells prior to (left panel) or after UV irradiation (right panel). Presented are the numbers of proteins that were identified from two independent experiments on cells that were fed pacFA for 1 h or 16 h. b) HeLa cells stably expressing RFP-tagged Golgi-resident mannisodase II were fed pacFA for 1 h and UV-irradiated, as indicated. Cells were fixed in methanol, extracted with chloroform/methanol/acetic acid, subjected to a click reaction with Alexa 488 azide, and then visualized by fluorescence microscopy. Bottom row: magnification of regions as indicated in the middle panel. Bars, 10 μm.

identified in non-UV-irradiated cells dropped by more than fourfold when cells were fed pacFA for 1 h instead of 16 h (from 214 to 49; see also Table S4 in the Supporting Information). In contrast, the number of lipid-crosslinked proteins identified in UV-irradiated cells was largely independent of whether cells were fed pacFA for 1 h or 16 h (267 and 202, respectively; Figure 3a and Table S5 in the Supporting Information). The bulk of these lipid-crosslinked proteins were identified twice (176 out of 293), in line with our finding that cells fed pacFA for 1 h or 16 h contain very similar collections of bifunctional lipids (Figure S5 in the Supporting Information). There was also a substantial overlap between lipid-crosslinked proteins identified in HeLa and CHO cells. In HeLa cells we found orthologs for 105 of the 253 lipid-crosslinked proteins isolated from CHO cells (42%; Figure S8c in the Supporting Information). Table S1 in the Supporting Information lists the top 99 high-confidence lipid-crosslinked proteins that were identified in both cell types and at least three times out of four independent experiments. Almost half of these proteins localize to the ER, whereas the second largest fraction (nearly 20%) comprises mitochondrial proteins. One-tenth of these proteins had previously been shown to handle fatty acids or fatty acid derived metabolites as their primary function. Among these were proteins that catalyze fatty acid import and catabolism in mitochondria (CPT2, ECHA), participate in phospholipid biosynthesis (CDIPT), transport (PITPB, STARD7), and turnover (ABHD6), mediate protein acylation in the ER (PIGS, GPI8), or control long-chain base levels for sphingolipid biosynthesis in the ER (ORML2, SGPL1).

The identification of two cytosolic phospholipid-transfer proteins, PITPB and STARD7, as high-confidence lipid-crosslinked proteins is of particular interest. To come in close contact with the photoactivatable group, these proteins must dip into the hydrophobic core of the bilayer or extract the bifunctional lipid from the membrane surface. While the precise cellular function of PITPB and STARD7 remains to be established, both proteins contain specific phospholipid binding sites and stimulate the monomeric transfer of particular phospholipids between membranes in vitro.[20–22] To gain further insight into the nature of the protein–lipid interactions by which PITPB and STARD7 were captured in our screen, the proteins were produced in *E. coli* and incubated with liposomes prepared from lipid extracts of HeLa cells that had been fed pacFA for 16 h. After the removal of unbound lipids by size-exclusion chromatography, the lipids bound to PITPB and STARD7 were identified by TLC and mass spectrometry. Consistent with their known lipid-binding specificities, PITPB interacted with both pacPC and pacPI, whereas STARD7 interacted exclusively with pacPC (Figures S9 and S10 in the Supporting Information). The protein-bound lipid fractions were devoid of pacFA or other bifunctional lipid species. Collectively, these data demonstrate the suitability of pacFA as a tool to identify protein–lipid interactions in living cells.

To test whether pacFA can also be used to visualize cellular protein–lipid interactions in situ, HeLa cells were fed pacFA for 1 h prior to UV irradiation. Omission of pacFA or UV irradiation served as controls (Figure 3b and Figure S11 in the Supporting Information). Next, the cells were fixed in methanol at −20 °C to precipitate cellular proteins and then extracted with chloroform to remove all lipids that were not covalently bound to proteins. Following a click reaction with Alexa 488 azide, the cells were analyzed by fluorescence microscopy. To minimize the metabolic incorporation of pacFA into palmitoylated proteins, labeling time was restricted to 1 h. As shown in Figure 3b, pacFA-fed UV-irradiated cells displayed a prominent reticular fluorescent staining of the cytoplasm that was highly reminiscent of the ER. This staining extended to the nuclear envelope and was abolished upon omission of either pacFA or UV irradiation. These results are consistent with our finding that nearly half of the high-confidence lipid-crosslinked proteins identified from pacFA-fed UV-irradiated cells reside in the ER (Table S1 in the Supporting Information).


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We next investigated whether pacFA can be used to map protein–lipid interactions in a multicellular organism such as the nematode *C. elegans*. L2 larvae were fed pacFA at 20 °C prior to UV irradiation. Total lysates were reacted with Alexa 488 azide and Alexa-labeled proteins were visualized by SDS-PAGE and in-gel fluorescence (Figure 4a). For the majority of these proteins, labeling was abolished when pacFA or UV irradiation was omitted. To visualize protein–lipid interactions in the entire organism, pacFA-fed and UV-irradiated L2 larvae were fixed in methanol and acetone. Non-crosslinked lipids were removed by chloroform extraction as described above and subjected to a click reaction with Alexa 488 azide. As shown in Figure 4b, pacFA-fed larvae displayed a bright fluorescent staining that was dependent on UV irradiation. The observed staining was not uniform and particularly prominent in intestinal epithelial cells as well as in muscle cells in the pharyngeal region. Both cell types displayed a reticular staining of the cytoplasm resembling the ER, as was also observed in pacFA-fed and UV-irradiated HeLa cells, which is in line with our finding that the majority of crosslinked proteins localize in the ER (Figure 2e). Weaker staining was observed in the hypodermis, whereas the gonads were essentially devoid of staining. Staining of the hypodermis, muscle and intestinal cells was completely abolished in pacFA-labeled worms when UV irradiation was omitted. The precise reason for this tissue-specific labeling pattern is unclear, but it deserves mention that intestinal epithelia and muscle cells generally harbor active fatty acid uptake systems.[23,24]

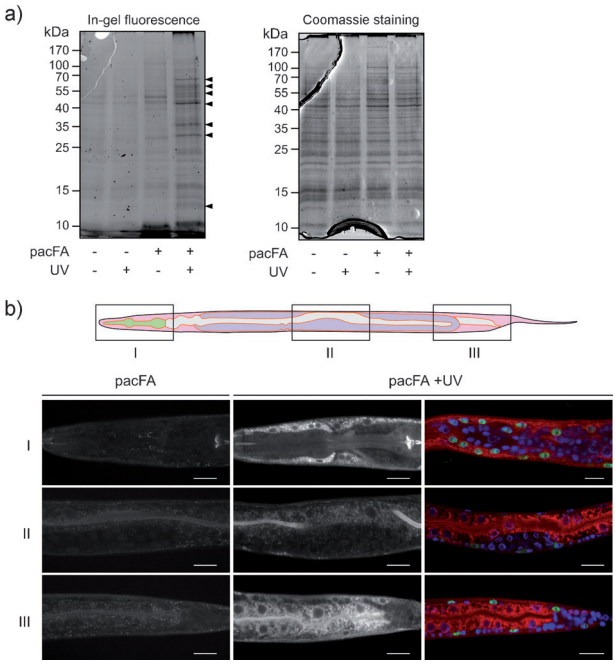


***Figure 4.*** Imaging of protein–lipid interactions in pacFA-fed *C. elegans*. a) Wild-type L2 larvae were fed pacFA and then subjected to UV irradiation, as indicated. Total cellular membranes were isolated, subjected to a click reaction using Alexa 488 azide, processed for SDS-PAGE, and then analyzed by in-gel fluorescence (left panel) or stained with Coomassie brilliant blue (right panel). b) *C. elegans* expressing a GFP-tagged muscle-specific transcription factor were fed pacFA and then subjected to UV irradiation, as indicated. Larvae were fixed in methanol and acetone, extracted with chloroform/methanol/acetic acid, subjected to a click reaction with Alexa 594 azide, stained with 4′,6-diamidino-2-phenylindole (DAPI) and then visualized by fluorescence microscopy. Images were taken from the head (I), mid-region (II), and tail (III), as schematically indicated. Left and middle panels show Alexa 488 fluorescence, whereas the right panels display a merge of Alexa 594, DAPI, and GFP fluorescence. Bars, 10 μm.

In summary, we have established the bifunctional fatty acid pacFA as a novel tool for the global profiling of protein–lipid interactions in living cells. Our approach combines the assets of previously reported photoactivatable and radioactive lipid precursors with click chemistry, thereby eliminating the drawbacks of radioactivity while providing novel opportunities for visual screens aimed at understanding how lipids function within their physiological context. The development of bifunctional precursors of sterol and sphingolipid biosynthesis is currently under way to complement this work and to enable a systematic profiling of proteins that bind sterols and sphingolipids, which constitute the other two main classes of membrane lipids. We show that the application of pacFA can be extended from cultured cells to *C. elegans*, thus providing a fresh starting point for a systematic investigation of protein–lipid interactions in the context of animal physiology and development.

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