

1 **PALP: An imaging method for detecting and quantifying polyunsaturated phospholipids**
2 **via peroxidation**

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19 **ABSTRACT**

20 **Polyunsaturated phospholipids are essential for multiple cellular functions; however, their**
21 **uncontrolled peroxidation leads to ferroptosis. Here we describe photochemical activation**
22 **of membrane lipid peroxidation (PALP), which uses localized laser pulses to induce lipid**
23 **peroxidation photochemically. While PALP bypasses enzymatic requirements for lipid**
24 **peroxidation, the resulting BODIPY-C11-based signal is largely correlated with local**
25 **polyunsaturated phospholipid concentration on membranes. This technique enables non-**
26 **invasive reporting of lipid unsaturation levels and sensitivity to ferroptosis in live cells.**

27 Ferroptosis, an iron-dependent, non-apoptotic cell death program, is a key contributor to tissue
28 damage occurring in a variety of acute organ injury as well as chronic degenerative diseases¹.
29 Ferroptotic cell death is driven by aberrantly accumulated oxidative damage of membrane
30 phospholipids possessing polyunsaturated fatty acyl (PUFA) chains, hence the cellular
31 susceptibility to ferroptosis is highly dependent on cellular PUFA-phospholipid content and
32 distribution. Recent research highlights that ferroptosis susceptibility varies dramatically among
33 different lineages and cell states². While targeting ferroptosis is emerging as an appealing strategy
34 for overcoming certain diseases, the heterogeneity in ferroptosis susceptibility among different
35 tissues and cell types will likely pose a challenge to achieve high efficacy with ferroptosis-inducing

36 agents. Therefore, there is a pressing need for techniques that enable rapid and non-invasive
37 assessment of cellular susceptibility to ferroptosis.

38 Multiple factors are known to contribute to the variable sensitivity to ferroptosis, including
39 availability of reactive iron, PUFA-phospholipid concentrations in membranes, activities of lipid
40 peroxidation-promoting enzymes, and cellular oxidative stress levels. Nonetheless, the rapid,
41 irreversible and pervasive nature of ferroptosis has prevented detailed characterizations of the
42 molecular processes of lipid peroxidation and ferroptotic cell death. Here, to enable visualization
43 of lipid peroxidation at high spatio-temporal resolution, we use high-power laser pulses to induce
44 local lipid peroxidation, the intensity of which is captured by fluorescent signals from oxidative
45 modification of the BODIPY-C11 probe³. This technique, termed photochemical activation of
46 membrane lipid peroxidation (PALP), bypasses the enzymatic reactions normally mediating lipid
47 peroxidation in cells and provides an approximate estimation for the cellular PUFA-phospholipid
48 levels.

49 To assess the dynamics of lipid peroxidation, we pre-treated the ferroptosis-susceptible 786-O
50 clear-cell renal cell carcinoma (ccRCC) cell line², with BODIPY-C11 (B-C11). B-C11 can be
51 oxidized by membrane associated lipophilic reactive oxygen species and switches from orange
52 (591nm) to green (503nm) fluorescence after oxidation⁴. We applied 5 pulses of 405 nm light from
53 a high-power laser source on a confocal microscope to a localized region in the cell over a 5
54 second period (**Fig. 1a**). The target cells were imaged for oxidized B-C11 (oxB-C11) signal for 25
55 seconds at 1 second intervals (**Fig. 1a**). We found that the 405 nm laser pulses induced strong
56 oxB-C11 signal in 786-O cells immediately following the laser pulses, and the signal gradually
57 decayed after reaching the peak intensity (normalized maximum intensity, PALP I-max) (**Fig. 1b-**
58 **c, Supplementary Video 1**).

59 We next assessed whether the oxB-C11 signal is indeed resulted from lipid peroxidation. Key
60 features of lipid peroxidation include its iron-dependence and ability to be quenched by lipophilic
61 radical-trapping antioxidants (RTAs)^{1,5}. Indeed, treatment with iron chelator ciclopirox olamine
62 (CPX), or lipophilic RTAs and ferroptosis inhibitors ferrostatin-1 (Fer-1) and liproxstatin-1 (Lip-
63 1)^{5,6,7} effectively reduced the PALP I-max signal in 786-O cells (**Fig. 1d-f, Supplementary Fig.**
64 **1a**), suggesting that PALP shares similar features of lipid peroxidation induced by chemical or
65 genetic inhibition of glutathione peroxidase 4 (GPX4)^{2,8}.

66 The subcellular origin of lipid peroxidation remains elusive, largely because the explosive signal
67 expansion and prompt membrane damage after inducing lipid peroxidation at a whole cell level
68 has prevented characterizations at high spatial-temporal resolution. Previous imaging analysis
69 revealed that the initial lipid peroxidation signal co-localizes with markers of the endoplasmic
70 reticulum (ER)⁹, highlighting the ER as the top candidate organelle where lipid peroxidation could
71 initiate. To assess whether lipid peroxidation indeed occurs in the ER, we labeled the ER in 786-
72 O cells with a fluorescent ER-tracker, and applied the laser pulses to regions both inside and
73 outside of the ER network. We found that while laser pulses induced a strong signal when applied
74 to ER-tracker marked areas, the signal is significantly weaker when applied to areas outside of
75 ER (**Fig. 1g**). This result suggests that lipid peroxidation likely requires the ER for initiation. In our

76 subsequent studies, we thus applied the laser pulses to the core ER structures, approximately
77 reported by the presence of strong non-oxidized B-C11 signal.

78 Lipid peroxidation can spread non-enzymatically via autoxidation or enzymatically via the
79 cytochrome P450 oxidoreductase (POR) and its associated redox partners in multiple neoplastic
80 cell types including 786-O ccRCC cells¹⁰, and through arachidonate lipoxygenases (ALOXs) in
81 certain other cellular contexts¹¹. To determine whether laser-induced lipid peroxidation is
82 dependent on POR activity, we applied laser pulses to *POR*^{-/-} single-cell 786-O clones we
83 previously generated¹⁰. As a control, we also analyzed cells that are depleted of KEAP1¹², which
84 promotes ferroptosis susceptibility in cancer cells by inducing degradation of NRF2, a master
85 transcriptional regulator of cellular antioxidant enzymes^{13,14}. As we found, POR-depletion did not
86 significantly alter PALP I-max signal, which was modestly reduced in KEAP1-depleted cells
87 (**Supplementary Fig. 1b**). This result suggests that POR activity is not required for PALP
88 induction. This likely reflects that high-power laser is bypassing POR to initiate lipid peroxidation
89 photochemically. On the other hand, this data also hints that POR is involved in early initiation,
90 rather than propagation of cellular lipid peroxidation reactions. The modest effects of KEAP1-
91 depletion suggest that activation of cellular antioxidant programs may only exert indirect effects
92 in attenuating PALP. Together, these results indicate that PALP is largely a photochemical
93 process that is independent of protein enzymatic activity. We hence speculated that the local
94 PUFA-phospholipid concentration might be the major rate-limiting factor regulating the PALP I-
95 max signal.

96 To investigate the relationship between cellular PUFA-phospholipid content and PALP signal, we
97 first depleted acyl-CoA synthetase long chain family member 4 (ACSL4) in ccRCC cells using
98 CRISPR/Cas9 (**Fig. 2a, Supplementary Fig. 2a**). ACSL4 catalyzes the conversion of long-chain
99 fatty acids into fatty acyl-CoA, and is a key requirement for PUFA-phospholipid synthesis as well
100 as a common mediator of ferroptosis susceptibility in multiple cellular contexts^{2,15,16}. Previous
101 lipidomic analysis showed that ACSL4-depletion selectively reduced the levels of cellular PUFA-
102 phospholipid levels, with compensatory upregulation of PUFA-triacylglycerides (TAGs)¹². As a
103 result, ACSL4-depleted cells exhibited significantly lower susceptibility to GPX4 inhibition-induced
104 ferroptosis (**Fig. 2b, Supplementary Fig. 2b**). Importantly, the PALP I-max signal was also
105 significantly suppressed in ACSL4-depleted cells (**Fig. 2c-e**), suggesting that high levels of PUFA-
106 phospholipids are necessary to potentiate strong PALP induction.

107 To explore whether PUFA-phospholipid upregulation is also sufficient to enable PALP induction,
108 we used a previously established *EPAS1*^{-/-} 786-O cell line model. In this model, ectopic
109 overexpression of hypoxia-induced, lipid droplet-associated protein (*HILPDA*), a direct HIF-2 α
110 (encoded by *EPAS1*) target gene, selectively restores the cellular PUFA-lipidome, including both
111 PUFA-phospholipids and PUFA-TAGs, in HIF-2 α -depleted cells². We found that HILPDA-
112 overexpressing cells exhibited significantly stronger PALP I-max signal compared with empty
113 vector-expressing cells (**Fig. 2f-g**), confirming that PUFA-lipids are rate-limiting for PALP
114 induction.

115 We next used chemical approaches to complement the genetic modulation of PUFA-phospholipid
116 levels. We pre-treated the human melanoma cell line WM-793¹⁷ with various synthetic fatty acids
117 for three days prior to PALP application. These fatty acids include monounsaturated fatty acid
118 (MUFA) oleic acid (OA, C18:1), and polyunsaturated fatty acids (PUFA) including arachidonic
119 acid (AA, C20:4 ω 6), docosapentaenoic acid (DPA, C22:5 ω 3), and docosahexaenoic acid (DHA,
120 C22:6 ω 3). This experiment revealed that treatment with AA, DPA or DHA, but not OA significantly
121 enhanced the PALP I-max signal; the extent of enhancement is correlated with the ferroptosis
122 sensitization activities of these fatty acids¹⁰ (**Fig. 2h-i, Supplementary Fig. 2c**). In another cell
123 line BFTC-909, a transitional renal cell carcinoma model, OA treatment even inhibited the PALP
124 I-max signal (**Supplementary Fig. 2d-e**). The PALP-suppressive effect of MUFA is consistent
125 with the recent identification of exogenous MUFAs as ferroptosis inhibitory agents¹⁸. Taken
126 together, these results support positive correlations among cellular susceptibility to ferroptosis,
127 the polyunsaturated-phospholipid levels, and the PALP I-max signal.

128 Recent research highlighted that PUFA-lipids play fundamental roles in shaping the ferroptosis
129 sensitivity in various cellular contexts^{2,9,19}. However, over-abundant PUFA-phospholipids in
130 membranes create a vulnerability to ferroptosis induction. While single-cell lipidomics awaits to
131 mature, there are limited tools to efficiently report the cellular PUFA-lipid levels in a cell. Here we
132 showed that high-power laser-induced lipid peroxidation (PALP) shares similar chemical and
133 biological properties as that induced by inhibition of GPX4 enzymatic activity. PALP bypasses the
134 requirements for peroxidation-promoting enzymes including POR, and is largely dictated by the
135 local PUFA-phospholipid levels in cellular membranes. Hence, the PALP technique provides a
136 practical and efficient approach to non-invasively evaluate the PUFA-lipid abundances in live
137 cells. We envision that PALP will be useful for studying the mechanisms of ferroptosis at high
138 spatio-temporal resolution. As a proof-of principle, we used PALP to learn that lipid peroxidation
139 requires the endoplasmic reticulum for initiation. Moreover, this technique could also be useful for
140 rapidly evaluating the PUFA-lipid levels in a heterogeneous cell population and contribute to
141 various fronts of lipid metabolism research.

142 **Methods**

143 **Cell lines and culture conditions.** 786-O, 769-P and WM-793 cells were cultured with RPMI
144 1640 (Gibco) medium. BFTC-909 cells were cultured with DMEM (Gibco) medium. All cell lines
145 were obtained from the Cancer Cell Line Encyclopedia (CCLE) distributed and authenticated by
146 the Broad Institute Biological Samples Platform and Genetic Perturbation Platform. All culture
147 media were supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin.
148 786-O-Cas9 cells expressing *KEAP1*- or *ACSL4*-targeting sgRNAs were established and
149 validated as previously described¹². *POR*^{-/-} single cell clones were generated and validated in
150 prior studies¹⁰. HILPDA-expressing *EPAS1*^{-/-} cells were generated as previously described².

151 **Chemicals.** ML210, RSL3, ferrostatin-1, liproxstatin-1 and ciclopirox olamine (CPX) were
152 obtained from commercial sources (Sigma-Aldrich, SML0521, SML2234, SML0583, SML1414,
153 and 1134030 respectively). CPX was used at 10 μ M. Ferrostatin-1 was used at 5 μ M. Liproxstatin-

154 1 was used at 1 μ M. BODIPY-C11 (Life Technologies) was reconstituted in DMSO, and added to
155 the cells 30 min prior to live-cell imaging at 5 μ M.

156 **Free fatty acid treatment.** Oleic acid (OA, C18:1), arachidonic acid (AA, C20:4),
157 docosapentaenoic acid (DPA, C22:5), and docosahexaenoic acid (DHA, C22:6) were purchased
158 from Cayman Chemicals and conjugated with fatty-acid free BSA (Sigma-Aldrich) using
159 previously described protocols²⁰, and treated cells at 20 μ M for 3 days prior to viability assays or
160 imaging analysis.

161 **Viability assays.** For cellular viability assays, cells were seeded in 384-well opaque white tissue
162 culture and assay plates (Corning) at 1000 cells/well. 18-24 hours after seeding, cells were treated
163 with compounds at the indicated concentrations for 48-72 hours. Cellular ATP levels were
164 quantified using CellTiter-Glo Luminescence Assay (Promega) on an Envision multi-plate reader
165 (PerkinElmer). Relative viability was normalized to the respective untreated condition of each cell
166 line using RStudio and plotted in PRISM 8 (GraphPad software). For data presentation, the mean
167 and standard deviation (s.d.) for the four biological replicates of each data point in a
168 representative experiment is presented. Sigmoidal non-linear regression models were used to
169 compute the regression fit curves.

170 **CRISPR/Cas9-mediated genome editing.** Cells were engineered for constitutive Cas9
171 expression using the pLX-311-Cas9 vector (Addgene 96924), which contains the blasticidin S-
172 resistance gene driven by the SV40 promoter and the SpCas9 gene driven by the EF1 α promoter.
173 sgRNA sequences were cloned into the pLV709 doxycycline-inducible sgRNA expression
174 vectors. Lentiviruses were generated from lentiviral Cas9 and sgRNA constructs in HEK-293T
175 packaging cells. Lipofectamine 2000 (Life Technologies) was used as transfection reagents to
176 deliver plasmids to cells following manufacturer's instructions. Second generation packaging
177 plasmids, including pMD2.G and pPAX2, was used for lentiviral production. Lentivirus titer was
178 briefly assessed with Lenti-X Go-Stix Plus (TakaraBio). Target cells were infected with lentiviruses
179 in the presence of 5 μ g/mL of polybrene (Millipore). Depending on the vector, infected cells were
180 selected with 2 μ g/mL of puromycin or 10 μ g/mL of Blasticidin S and propagated for further
181 analysis. Cells transduced with doxycycline-inducible constructs were treated with 1 μ g/ml of
182 doxycycline (Sigma-Aldrich) for 7-14 days prior to gene-knockout validation using immunoblotting.
183 Sequences of sgRNAs used in CRISPR experiments are: *ACSL4*-sg1,
184 GCATCATCACTCCCTTAGGT; *ACSL4*-sg2, GTGTGTCTGAGGAGATAGCG.

185 **Immunoblotting.** Adherent cells were briefly washed twice with ice-cold PBS and lysed with 1%
186 SDS lysis buffer containing 10 mM EDTA and 50 mM Tris-HCl, pH 8.0. Lysates were collected,
187 briefly sonicated, then incubated at 95 $^{\circ}$ C for 10 min and the protein concentrations were
188 determined using the BCA Protein Assay kit (Pierce) following manufacturer's instructions.
189 Calibrated samples were diluted with 4x lithium dodecyl sulfate (LDS) sample buffer (Novus),
190 separated by SDS-PAGE using NuPAGE 4–12% Bis-Tris protein gels (Novus), and transferred
191 to nitrocellulose or PVDF membranes by an iBlot2 protein-transfer system (Thermo Fisher
192 Scientific). Membranes were blocked with 50% Odyssey blocking buffer (LiCor) diluted with 0.1%
193 Tween-20-containing Tris buffered saline (TBST) and immunoblotted with antibodies against
194 *ACSL4* (Abcam, ab155282, produced in rabbit, used at 1:1000 dilution) and β -Actin (8H10D10,

195 no. 3700 and 13E5, no. 4970, Cell Signaling Technologies, used at 1:5,000 dilution). Membranes
196 were then washed with TBST and incubated with IRDye 800CW goat-anti-Rabbit or 680RD
197 donkey-anti-Mouse secondary antibodies (LiCor). Immunoblotting images were acquired on an
198 Odyssey equipment (LiCor) according to the manufacturer's instructions, and analyzed in the
199 ImageStudio software (LiCor). β -Actin was used as a loading control.

200 **Laser imaging and data analysis.** Imaging was performed on an Andor Revolution Spinning
201 Disk Confocal, FRAPPA and TIRF microscope. Prior to high-power laser application, steady state
202 images were acquired to visualize the distributions of reduced and oxidized BODIPY-C11 in cells
203 respectively. Each confocal laser was set at 3mHz and standard gain with a 200 ms exposure.
204 FRAPPA bleaching was done with a 405 nm laser for 5 pulses and the 488 nm confocal channel
205 was used to collect images for another 25 seconds following the laser pulse. Images are acquired
206 using the Metamorph software associated with the equipment. Subsequently, image analysis was
207 performed using Fiji ImageJ (1.52P). ROIs were imported from metamorph to locate laser
208 bleaching regions. Each series measurement was analyzed using ImageJ ROI manager multi-
209 measurement tool at a 10 px radius around the region of interest. Data was normalized to time
210 point before bleaching occurred and the resulting plot was fit with a non-linear regression to find
211 I-max (Y0), T50, Plateau and T20 in Prism 8 (GraphPad).

212 **Software and statistical analysis.** Data are generally expressed as mean \pm s.d. unless otherwise
213 indicated. No statistical methods were used to predetermine sample sizes. Statistical significance
214 was determined using a two-tailed, unpaired student's T-test using Prism 8 software (GraphPad
215 Software) unless otherwise indicated. Statistical significance was set at $p \leq 0.05$ unless
216 otherwise indicated. Figures are finalized in Adobe Indesign and Illustrator.

217 **Data and Code Availability Statement**

218 Raw videos showing the dynamics of lipid peroxidation signal in wildtype 786-O cells are provided
219 as a **Supplementary Video**. All original data and computational code that support the findings of
220 this study are available upon request.

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227 **Author contributions**

228 Y.Z. initiated and conceived the project with input from Y.H. and L.Y. S.L.S. supervised the project.
229 E.G. and Y.Z. performed the experiments and data analyses. W.S. assisted the imaging analyses.

230 **Competing financial interests**

231 S.L.S. serves on the Board of Directors of the Genomics Institute of the Novartis Research
232 Foundation (“GNF”); is a shareholder and serves on the Board of Directors of Jnana Therapeutics;
233 is a shareholder of Forma Therapeutics; is a shareholder and advises Kojin Therapeutics, Kisbee
234 Therapeutics, Decibel Therapeutics and Eikonizo Therapeutics; serves on the Scientific Advisory
235 Boards of Eisai Co., Ltd., Ono Pharma Foundation, Exo Therapeutics, and F-Prime Capital
236 Partners; and is a Novartis Faculty Scholar. Kojin Therapeutics in particular explores the medical
237 potential of cell plasticity related to ferroptosis. Other authors declare no conflict of interest
238 relevant to this study.

239 **Additional information**

240 Further information and requests for resources and reagents should be directed to the
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242 (yzou@broadinstitute.org).

243

244 **Figure legends**

245 **Figure 1. Targeted laser pulses induce localized lipid peroxidation in live cells.**

- 246 a. Schematic diagram describing the procedures of the photochemical activation of
247 membrane lipid peroxidation (PALP) technique. BODIPY-C11 at a concentration of 5 μM
248 was added to the cells approximately 30 min prior to imaging. Then a point on the cell
249 was targeted by 5 pulses of the 405 nm laser. Each pulse lasts for 1 second. The 488
250 nm channel intensity was then recorded for 25 seconds at 1 second interval to monitor
251 the oxidized BODIPY-C11 signal.
- 252 b. Fluorescent images showing the time course of the reduced and oxidized BODIPY-C11
253 signal before and after the application of 405 nm laser pulses in wildtype 786-O cells.
254 Scale bar indicates 10 μm .
- 255 c. Schematic diagram showing the parameters used to describe the dynamics of lipid
256 peroxidation signal in a representative PALP experiment. All oxBODIPY-C11 signals are
257 normalized by deducing the pre-laser intensity from the detected signals. I-max,
258 normalized maximal intensity, T_{20} , time (sec) it takes to reach 20% reduction from I-max
259 signal. T_{50} , time (sec) it takes to reach 50% reduction from I-max signal. I-plateau,
260 normalized signal intensity when the oxBODIPY-C11 signal largely plateaued.
- 261 d. Quantifications of the PALP signal intensity from time-lapse imaging of 786-O cells
262 treated with ciclopirox olamine (CPX), ferrostatin-1 (Fer-1) and liproxstatin-1 (Lip-1) and
263 stimulated with laser pulses. 5 cells were measured for each condition, and error bars
264 indicate mean \pm s.d.
- 265 e. Box-scatter plots showing the PALP parameters of 786-O cells treated with ciclopirox
266 olamine (CPX), ferrostatin-1 (Fer-1) and liproxstatin-1 (Lip-1).

- 267 f. Representative fluorescent images showing the I-max (0 sec) post the application of
268 laser pulses to 786-O cells treated with the indicated compounds. Scale bar indicates 15
269 μm . Green, oxidized BODIPY-C11 signal; red, reduced BODIPY-C11 signal.
- 270 g. Image representation of endoplasmic reticulum (ER) staining and PALP lipid
271 peroxidation intensities within and outside the ER in 786-O cells. Quantifications of I-max
272 are on the right.

273 **Figure 2. Photochemically activated lipid peroxidation signal correlates with**
274 **polyunsaturated phospholipid levels.**

- 275 a. Immunoblot analysis of ACSL4 protein levels in 786-O cells expressing non-targeting
276 negative control sgRNA (sg-NC) or *ACSL4*-targeting sgRNAs. β -actin was used as a
277 loading control.
- 278 b. Viability curves of 786-O cells expressing sg-NC or sgRNAs targeting *ACSL4* treated
279 with indicated concentrations of GPX4 inhibitors ML210 or RSL3 for 48h. n=4; error bar,
280 mean \pm s.d.
- 281 c. Representative fluorescent images showing the I-max (0 sec) post the application of
282 laser pulses to 786-O cells expressing sg-NC or *ACSL4*-targeting sgRNA. Green,
283 oxidized BODIPY-C11 signal; red, reduced BODIPY-C11 signal.
- 284 d. Quantifications of the PALP signal intensity from time-lapse imaging of 786-O cells
285 expressing sg-NC or *ACSL4*-targeting sgRNA after laser activation. 5 cells were
286 measured for each condition, and error bars indicate mean \pm s.d.
- 287 e. Box-scatter plots showing the PALP parameters of 786-O cells expressing sg-NC or
288 sgRNAs targeting *ACSL4*. Two-tailed unpaired T-test, *, $p < 0.05$,
- 289 f. Box-scatter plots showing the PALP parameters (I-max and I-plateau) of *EPAS1*^{-/-} 786-O
290 cells expressing empty vector (EV) or *HILPDA* cDNA. Two-tailed unpaired T-test, **,
291 $p < 0.01$.
- 292 g. Representative fluorescent images showing the I-max (0 sec) post the application of
293 laser pulses to *EPAS1*^{-/-} 786-O cells expressing empty vector (EV) or *HILPDA* cDNA.
294 Green, oxidized BODIPY-C11 signal; red, reduced BODIPY-C11 signal.
- 295 h. Box-scatter plots showing the I-max of PALP signals in WM-793 melanoma cells treated
296 with vehicle (veh) or indicated free fatty acids. Two-tailed unpaired T-test, **, $p < 0.01$, ***,
297 $p < 0.001$.
- 298 i. Fluorescent images showing the I-max (0 sec) post the application of laser pulses to
299 WM-793 cells treated with vehicle (Veh, 5% BSA) or BSA conjugated arachidonic acid
300 (C20:4). Scale bar indicates 10 μm . Green, oxidized BODIPY-C11 signal; red, reduced
301 BODIPY-C11 signal.

302 j. Schematic diagram summarising the applications of PALP for detecting and quantifying
303 polyunsaturated phospholipid levels via photochemically-induced lipid peroxidation in
304 live cells. PUFA, polyunsaturated fatty acyl-.

305 **Supplementary Figure Legends**

306 **Supplementary Figure 1. Targeted laser pulses induce localized lipid peroxidation in live** 307 **cells.**

308 a. Viability curves for 786-O cells treated with ciclopirox olamine (CPX), Lip-1, or Fer-1 and
309 ML210 for 48h. n=4; error bar, mean±s.d.

310 b. Box-scatter plots showing the PALP parameters (I-max and I-plateau) of 786-O-Cas9
311 cells expressing sg-NC or sg-*KEAP1*, or a *POR*^{-/-} 786-O single-cell clone.

312 **Supplementary Figure 2. PALP signal correlates with cellular polyunsaturated** 313 **phospholipid levels.**

314 a. Immunoblot analysis of ACSL4 protein levels in 769-P cells expressing negative control
315 sgRNA (sg-NC) or *ACSL4*-targeting sgRNAs. β -actin was used as a loading control.

316 b. Viability curves of 769-P cells expressing sg-NC or *ACSL4*-targeting sgRNAs treated
317 with indicated concentrations of GPX4 inhibitors ML210 or RSL3 for 48h. n=4, error bar,
318 mean±s.d.

319 c. Viability curves for WM-793 cells treated with vehicle or indicated fatty acids, together
320 with DMSO, Lip-1 or Fer-1 and indicated concentrations of ML210 or RSL3 for 48h. n=4,
321 error bar, mean±s.d.

322 d. Box-scatter plots showing the I-max of PALP signals in BFTC-909 transitional renal cell
323 carcinoma cells treated with indicated free fatty acids. Two-tailed unpaired T-test, **,
324 $p < 0.01$, ***, $p < 0.001$.

325 e. Representative fluorescent images showing the I-max (0 sec) post the application of
326 laser pulses to BFTC-909 cells treated with vehicle or oleic acid (C18:1). Green: oxidized
327 BODIPY-C11 signal.

328 **Supplementary Video 1.** Time-lapse imaging of oxidized BODIPY-C11 signal in 786-O cells
329 stimulated with PALP. White signal, oxidized BODIPY-C11 signal. The time scale is accelerated
330 at 3x speed.

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Figure 1

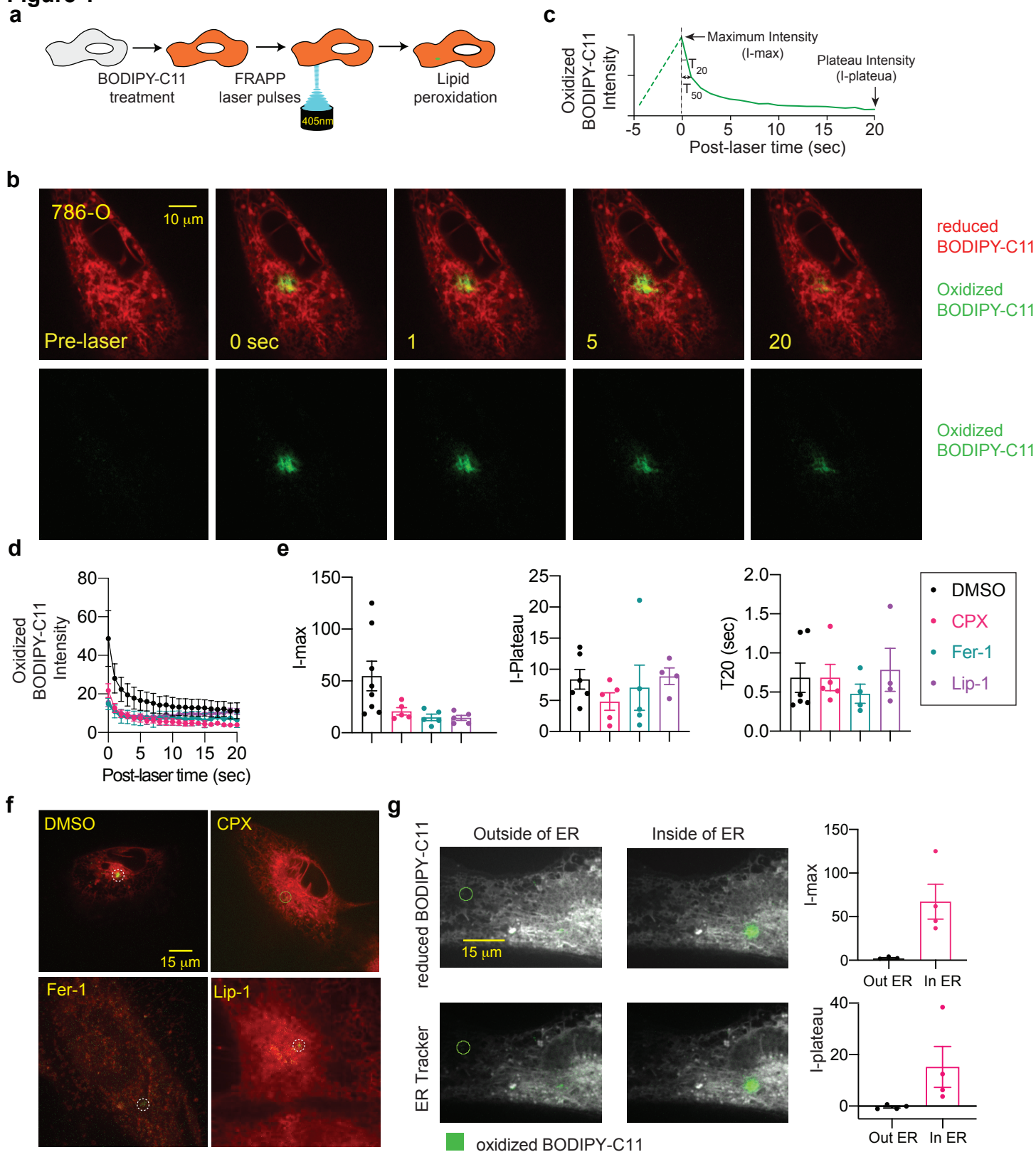
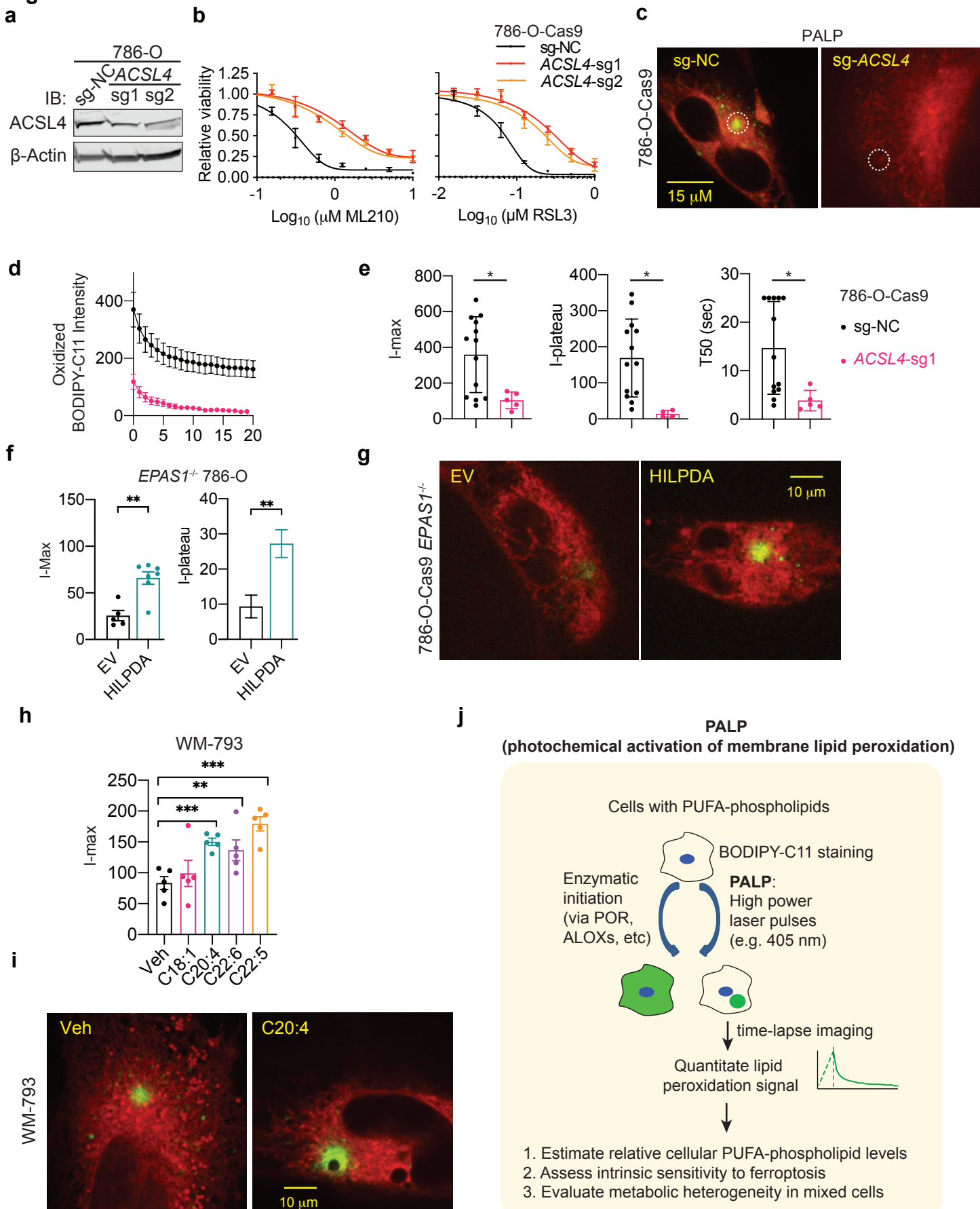
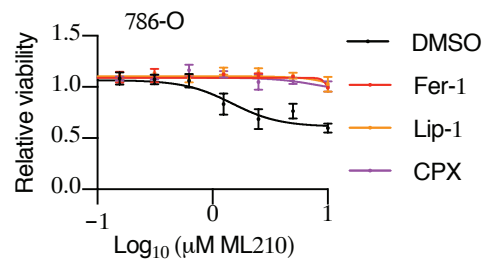


Figure 2

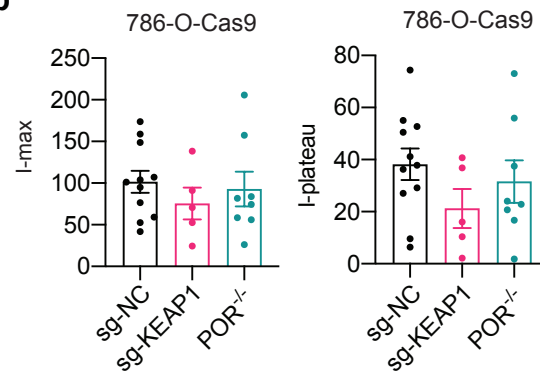


Supplementary Figure 1

a



b



Supplementary Figure 2

