pHLARE: A New Biosensor Reveals Decreased Lysosome pH in Cancer Cells

Bradley Webb, Francesca Aloisio, Rabab Charafeddine, Jessica Cook, Torsten Wittmann, and Diane Barber

Corresponding author(s): Diane Barber, University of California, San Francisco

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
RE: Manuscript #E20-06-0383  
TITLE: pHLARE: A New Biosensor Reveals Decreased Lysosome pH in Cancer Cells

Dear Dr. Barber:

Two experts have now reviewed your manuscript and both of them found the new method presented to be of interest and worth publishing. Both reviewers have, however, listed a number of relevant concerns that must be addressed prior to publication.  
Of special importance is to re-write the introduction and discussion in a more balanced fashion and to explain the experimental settings more precisely.  
In order to be able to conclude anything on cancer-and Ras-associated changes in lysosomal pH, the authors should include a large panel of non-transformed and transformed cell lines and show the effect of Ras in different non-transformed cell lines, respectively. The lysosomal pH in cancer cells being a controversial issue, it could be more informative to measure the pH in juxtanuclear and peripheral lysosomes separately.

Sincerely,

Marja Jaattela  
Monitoring Editor  
Molecular Biology of the Cell

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Dear Dr. Barber,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter"). Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker  
Journal Production Manager  
MBoC Editorial Office  
mbc@ascb.org
Reviewer #1 (Remarks to the Author):

In this manuscript, Webb et al. introduce an elegant method to measure lysosomal pH that complements a number of other existing and widely used methods. The method is based on the generation of a chimeric construct consisting of LAMP-1 tagged on its luminal side with sfGFP, a pH-sensitive derivative of GFP, and with mCherry on the cytosolic side. sfGFP fluorescence decreases with acidic pH and is normalized with cytosolic tagged RFP.

The method is rationally designed and seems to work effectively. The authors next used it to perform some measurements in cells treated with torin, an inhibitor of the mTORC1 complex, and compared the lysosomal pH of several normal and transformed cells. There are a number of issues that the authors need to address and several statements that are seemingly incorrect or incomplete, which could bias the readers.

Major:
1. The authors acknowledge in the Discussion that a fraction of the LAMP is in fact at the surface of cells, and this is clearly visible in the images. The mistargeting should be acknowledged early in the manuscript and the fraction of the LAMP-1 not in lysosomes should be quantified. Even if the fraction is comparatively small, the brighter fluorescence of the plasmalemmal probe can distort the readings and its contribution would be greater in thinner regions of the cells.
2. The authors used stable transfectants in some instances and transients in others. The amount of LAMP mistargeted to the membrane is very likely to be greater when markedly overexpressed. As before, the authors should acknowledge and discuss the confounding contribution of plasmalemmal (or early and late endosomal) LAMP to the putative lysosomal determinations.
3. sfGFP and mCherry must photobleach at different rates. This would introduce additional distortion to the measurements. Again, this caveat is not acknowledged or discussed.
4. Based on Figure 1E, the authors state overtly and definitively that the probe (termed pHLARE) does not undergo proteolysis in the lysosomes. However, the figure shows two rather distinct bands that differ by ~30 kDa, which is curiously similar to the m.w. of GFP. On what basis did the authors eliminate the occurrence of proteolysis and how are the two bands accounted for? Again, an explicit discussion of these observations is warranted.
5. What is the signal-to-noise ratio of the sfGFP fluorescence in acidic lysosomes? This is never stated or discussed and is essential for reliable measurements. The authors indicate that at physiological pH the organelles are not always identifiable based on the green channel, suggesting that the signal is very poor.
6. A large fraction of the manuscript is devoted to the torin and cancer cell data. The toin finding is not new and the differential pH of lysosomes of normal vs. transformed cells is inconsistent and debated in the literature. More importantly, no mechanism is proposed or investigated to account for any of these effects. These data should be omitted or at least greatly de-emphasized.

Other comments
1. The authors state that current reagents such as dextrans are limited because they are taken up by phagocytosis and are (therefore?) not exclusively localized in lysosomes. To my knowledge dextrans are taken up by endocytosis or macropinocytosis, but not by phagocytosis, which explains why they have been used in cells that are not phagocytic. Moreover, the dextrans can be restricted to lysosomes by using judicious pulse-chase protocols. In fact, such protocols ensure better localization to lysosomes than can be achieved by LAMP targeting, since LAMP is widely acknowledged to be present in late endosomes and, as the authors show, mistargets to the plasma membrane also. A more balanced discussion of these issues is necessary.
2. Oregon green 498 and 514 dextran conjugates can in fact be used for ratiometric imaging and do not need to be used in conjunction with another fluorophore. Oregon green 488 can be excited at both 490 nm and 440 nm wavelengths and Oregon green 514 can be excited at both 510 nm and 450 nm using a single emission wavelength for ratiometric pH imaging.
3. The authors note that lysosome size increases when using chloroquine to alkalize whereas bafilomycin does not. While bafilomycin increases the pH by inhibiting the proton pump, there is literature suggesting that chloroquine impairs lysosome function and can induce lysosomal damage and even permeability. For example, it can directly inhibit palmitoyl-protein thioesterase 1 (PMID: 30442709). It is possible that chloroquine-induced lysosomal damage results in enlarged/swollen lysosomes. It is also unclear how stimulation of lysosome biosynthesis by TFEB would account for the size change. Why would the new lysosomes be any bigger?
4. Why were cells treated with ammonia for 18 hrs, when the weak base alkalines in seconds?
5. Is Lysosensor in fact a dextran conjugate, as stated?
6. The differential pH of normal cells and those of patients with neurodegenerative diseases is far from well established and has been debated and disproven in some cases. A more balanced presentation of the field should be offered.

Reviewer #2 (Remarks to the Author):

The manuscript of Webb et al presents a new method to analyze lysosomal pH using a genetically encoded ratiometric biosensor consisting of sfGFP and mCherry fused to LAMP-1. The authors verify the lysosomal presence of the sensor and then use the biosensor to compare lysosomal pH in untransformed and cancerous cells. The study is very interesting however, the methodological part needs to be strengthened to convince of the quality of the method and the manuscript should benefit from a better focus.
Major:

My first concern is the choice of cell type. Are the RPE cells pigmented? Could melanin affect the fluorescence signal? Even if the cells are not pigmented they might still contain melanosomes. Since melanosomes are LRO (lysosome related organelles), they contain several lysosomal proteins e.g. LAMP proteins. Could pHLARE in transfected cells be expressed in melanosomes as well? The pH of melanosomes increase during maturation. When studying the frequency of pH in single lysosomes (Figure 2D), there are actually “two peaks”: one at pH 4-4.5 and one at pH 5.75-6. Could they represent two different organelles - lysosomes and (immature) melanosomes? Separating melanosomes from lysosomes are challenging but I recommend the authors to measure if melanin is present, as well as check the expression of melanosome protein markers e.g., NKI/beteb (early melanosomes) and tyrosinase (late melanosomes).

Images showing the colocalization between sfGFP and mCherry is not convincing. In figure S1, overlay images seem to have vacuolar structures with only sfGFP (blue) and only with mCherry (magenta). The fluorophores are situated on either side of the lysosomal membrane - is there a 100% overlap? What is the maximum distance between two fluorophores that are presented as one pixel (i.e., colocalized in images)? Clear description of the optical limitation of the microscope, and the resolution of the images should be presented.

Figure 1B: Is mCherry specific for lysosomes? The image shows bright staining at the plasma membrane. The same comment for Figure 2A in which sfGFP seems to be present unspecific.

It is not clear to me how the authors calibrate each set of experiment. Is a new “standard curve” constructed for every experiment? In materials and method, pH 5.0 and 6.5 nigericin samples is mentioned. Is this the day-to-day calibration? To construct a standard curve at least three points should be analyzed to make sure that the slope is correct. What is the variation in fluorescence ratio between different experiments? What is the transfection degree in transiently transfected cells? Are all cells expressing equal level of the sensor?

Several different cell lines are used and pH compared; how is the lysosomal pH calibrated between different cell lines? Is one calibration used for all different cell lines or is a separate standard curve constructed for each cell line? Please clarify.

It is not clear when the pH values are presented as median or as mean. Box and whisker plots are median but in Figure 1D pH is presented as means. Moreover, for example page 7, line 17-18 "The two calibration methods gave median pH values that were not significantly different (local background calibration \( pH_{lys} = 4.95 \) {plus minus} 0.22 (mean and SD)...." Another example on page 10 " We used RPE cells stably expressing pHLARE and quantitative measurements to show that Torin-1, [...]decreased pHlys from 5.22 {plus minus} 0.10 in controls to 4.63 {plus minus} 0.09 (mean {plus minus} s.e.m., 4 cell preparations) (Fig. 4D)." Similar in Figure 5. Please, clarify how median and mean values are used.

The authors argument that the pKa=4.5 of mCherry makes it insensitive to changes in cytosolic pH and therefore allows ratiometric analysis. However, during calibration with nigercin at pH 4.4 (slightly below pKa) the fluorescence of mCherry seems to be affected (figure 2B). How is this affecting the standard curve? This needs to be considered and discussed.

Transfections always affect the characteristics of the cell. In our hands, transfection with GFP-LAMP1 reduces the ability of lysosomes to move. In these experiments two fluorophores is fused to LAMP1. Is the locomotion of lysosomes affected? How is the endogenous protein levels of e.g., LAMP1 and LAMP 2 in transfected cells compared to wt? Some experiments to illustrate if the pHLARE transfected cells are functionally comparable to wt or if they differ, would be good to show.

Figure 1E: An “unspecific band” is used as loading control. Why not use a proper housekeeping protein as loading control? Figure 3B: The image should be larger and a magnification bar added. Markings in the image presenting how the different areas are selected should be added (nucleus, perinuclear lysosomes, lysosomes in distal membrane protrusions). Most perinuclear lysosomes appear in clustered. Is it a risk that these are deselected and a bias introduced when only well separated lysosomes are analyzed?

On page 8 the Figures are presented in random order: Fig 3A - Fig 5B and G - Fig 5E - Fig 5A - Fig 2D. Rewrite the text or rearrange the figure so that the results can be presented sequentially.

Figure S3: Why is cells pre-incubated during 18 h with NH4Cl? Most protocols use 30 min.

Figure 4B: Concentration of chloroquine is higher than in most protocols - could such high concentration cause lysosomal membrane permeabilization? The increase in lysosome size shown in Figure 4A could then be interpreted as lysophagy of damaged lysosomes.
Figure 4D-E: I do think the analysis of mTOR effect of pH is interesting but the results must be accompanied with a more thorough analysis of the cellular effect of Torin-1 exposure. Is autophagy altered, is the size and localization of lysosomes changed?

Figure 4F: The authors should prove by a proliferation marker that the cells are truly quiescent.

Figure 5 F and 5G is not included in the manuscript.

I think that the authors should streamline their manuscript. Now, the Results contain a lot of references; passages that is "introduction", several comparison of previously determined lysosomal pH and explanations (could rather be part of the discussion). Then most of the discussion is concentrated on comparing lysosomal pH in different cancer cell lines and pure speculations of the impact for invasion and drug resistance with help of references. I think that the focus of the manuscript is lost. (If the correlation between invasion and lysosomal pH is such interesting, an invasion/migration experiment is quite easy to perform since all cell lines are available.) However, I think that pros and cons of the method should be in focus here and the discussion on impact of lysosomal pH in cancer cell lines reduced.

The supplement showing the step-by-step instruction for pH calculation is very good. However it is 109 pages; several are blank, some only denote NaN and at the end several tables that are poorly explained. Please delete unnecessary material or label correctly.

The MBoC checklist demand that the method of cell line authentication and the frequency of testing for mycoplasma contamination is presented. This information is not in the manuscript.

Minor:

Figure 1B and C - the colors should be explained (compare figure 2B where sfGFP (green) and mCherry (magenta) is clarify). Colocalization presented as white should also be described

What are the small yellow markings in figure 3B and 5A? If of importance it should be enlarged and explained, if not important deleted.

The typesetting must be thoroughly checked; superscript/subscript should be used in all chemical formulas; prefix of Greek letters must be checked.
Figure 2: the letter E is lacking in the panel
Figure 4: the letter C I lacking in the panel
There are two supplements numbered S3 - one should be S4
Page 7, line 17: "We validated this analysis by comparing pHlys measurements in six RPE cells (Fig. 2D). Must be Fig 2E?

Font size of the y-axis (pH) is often too small
We are submitting a revised manuscript entitled **pHLARE: A New Biosensor Reveals Decreased Lysosome pH in Cancer Cells** for review as an article in MBoC. We thank the Monitoring Editor and Reviewers for their suggestions, which we addressed for a stronger manuscript. Our response to their requests and comments are outlined below. We hope that these changes make the work acceptable for publication.

Sincerely,

Diane L. Barber, PhD
Professor and Chair
Leland A. and Gladys K. Barber Endowed Chair in Dentistry
Department of Cell and Tissue Biology

**Monitoring Editor**

…*the authors should include a large panel of non-transformed and transformed cell lines and show the effect of Ras in different non-transformed cell lines.*

As requested, our revised manuscript includes new data on lysosome pH (pHlys) in a number of additional cell lines, including CCL39 fibroblasts untransformed (Fig. 3)
MDCK cells stably expressing K-RasV12 (new Fig. 5D)
Mia PaCa-2 pancreatic cancer cells (new Fig. 6A)
HCT116 colon cancer cells (new Fig. 6B)
H1299 lung cancer cells (a new Fig. 6B)

With these new data, we now include quantitative analysis of pHlys in 6 lines of untransformed cells and 11 lines of transformed cells, which we believe constitutes a substantial panel of cells from different tissue types, different genetic signatures, and different phenotypes.

We also include new data on intracellular pH (Fig. 6C) that address the question on a relationship between cytoplasmic and lysosomal pH, which our data indicate are not related.

*The lysosomal pH in cancer cells being a controversial issue, it could be more informative to measure the pH in juxtanuclear and peripheral lysosomes separately.*

As requested, we analyzed peripheral compared with total pHlys in MDA-MB-157 breast cancer cells. However, despite differences we measured in peripheral compared with perinuclear pHlys in RPE cells (Fig. 3B) we found no differences in pHlys between localized populations of lysosomes in MDA-MB-157 cells, which we
now indicate in the text. Additionally, despite quantified more peripheral lysosomes in MDA-MB-157 cells compared with untransformed breast MCF10A cells, this was not seen in pancreatic, colon, or glioblastoma cells, as indicated in new images included in Fig. 5A and B. The lack of more peripheral lysosomes in these cancer cells is described in the text.

Reviewer 1

Major

1. The authors acknowledge in the Discussion that a fraction of the LAMP is in fact at the surface of cells and this is clearly visible in the images. This mistargeting should be acknowledged early in the manuscript and the fraction of the LAMP-1 not in lysosomes should be quantified. Even if the fraction is comparatively small, the brighter fluorescence of the plasmalemmal probe can distort the readings and its contribution would be greater in thinner regions of the cells.

Reviewer 1 is correct that a fraction of pHLARE localizes to the cell membrane. In the previous version of the manuscript, we did not only acknowledge this in the discussion, but also described this in the results section where we presented our pHlys analysis strategy. We revised the text to make this even clearer. It is most important to note (and we discuss this in detail in the results) that our pHLARE analysis takes this cell membrane localization into account by subtracting a local background (i.e. the mean fluorescence of a ring of pixels around each lysosome object) from each corresponding lysosome fluorescence measurement. Thus, the cell membrane localization does not contribute to the measured lysosome signal.

The reviewer is correct though that we had not quantified how much of the pHLARE signal is in lysosomes and how much is not. We now include this measurement by modifying the quantification of co-localization with SiR lysosome, a far-red labelled pepstatin A that binds to cathepsin D. This new quantification also better addresses several other reviewers’ questions and is more like our subsequent pHLARE analysis strategy. In our hands, SiR lysosome is very specific with essentially no fluorescence signal in the cytoplasm. We therefore now use the SiR lysosome channel to define lysosomes by thresholding (Fig. 1E; Fig. S1) and then ask how much pHLARE fluorescence is contained within these SiR lysosome-defined objects compared with signal in the remainder of the cell (Fig. 1F). This analysis (that also considers the cell membrane signal underneath lysosomes) shows approximately half of the pHLARE mCherry signal within SiR lysosome-defined objects.

2. The authors used stable transfectants in some instances and transients in others. The amount of LAMP mistargeted to the membrane is very likely to be greater when markedly overexpressed. As before, the authors should acknowledge and discuss the confounding contribution of plasmalemnal (or early and late endosomal) LAMP to the putative lysosomal determinations.

Again, pHLARE cell membrane localization should contribute very little to lysosome pH measurements because a local background is subtracted from each lysosome object signal. The reviewer is likely correct that mistargeting to the cell membrane might increase at very high expression levels. However, at the expression levels typically used here, the fraction of
pHLARE localization within lysosomes was largely independent of the expression level (i.e. the mean pHLARE mCherry fluorescence signal in the cell; see new Fig. S1D).

3. sfGFP and mCherry must photobleach at different rates. This would introduce additional distortion to the measurements. Again, this caveat is not acknowledged or discussed.

We agree that sfGFP and mCherry photobleach at different rates, with sfGFP bleaching slightly faster. However, during our limited image acquisition times (3 images at 10s intervals each for steady state, higher pH nigericin, and lower pH nigericin) there is minimal photobleaching of sfGFP and mCherry. New photobleaching data are included in Fig. S2A and discussed in the text.

4. Based on Figure 1E, the authors state overtly and definitively that the probe (termed pHLARE) does not undergo proteolysis in the lysosomes. However, the figure shows two rather distinct bands that differ by ≈30 kDa, which is curiously similar to the m.w. of GFP. On what basis did the authors eliminate the occurrence of proteolysis and how are the two bands accounted for? Again, an explicit discussion of these observations is warranted.

As requested, we include in the text that based on immunoblotting with RFP antibodies, there is no apparent degradation of the probe. As requested by Reviewer 2, a revised Fig. 1 includes new immunoblot to include a GAPDH probe to show equal loading. As we indicate in the text, two bands for the pHLARE signal are likely due to glycosylation level that can cause a 30 kDa difference. We often see this is immunoblot for the Na-H exchanger, with signals at 75 and 100 kDa from differences in glycosylation. The RFP immunoblot does show bands below the pHLARE signal; however, because these are also seen in the first lane of lysates from wild-type RFP cells not expressing pHLARE we interpret them to be non-specific and indicate them with asterisks. Importantly, the data indicate that full-length pHLARE abundance does not change with nigericin calibration buffers. Additionally, if some sfGFP, not indicated in our immunoblot, is missing, our analysis controls for this on a cell-by-cell basis because we make a specific calibration curve for each cell. So, even if some sfGFP is missing that would be corrected for in the nigericin calibration.

5. What is the signal-to-noise ratio of the sfGFP fluorescence in acidic lysosomes? This is never stated or discussed and is essential for reliable measurements. The authors indicate that at physiological pH the organelles are not always identifiable based on the green channel, suggesting that the signal is very poor.

Our statement that it was hard to identify lysosomes in the sfGFP channel is based on the low contrast between sfGFP in the cell membrane (at neutral pH) and sfGFP in the lysome (at low pH). Thus, intensity-based thresholding of the sfGFP channel does not work well in control cells as for example a membrane fold will have the same or higher intensity as a lysosome in the sfGFP channel. It should be noted that sfGFP thresholding had only been included in the previous manuscript version to analyze co-localization with a lysosome marker and is not done in the pHLARE analysis algorithm. As outlined above, we have now replaced the co-localization analysis with an improved analysis that only relies on defining lysosomes in the SiR-lysosome channel and then measure fluorescence in the pHLARE channels.
Determining SNR in fluorescence microscopy is not straightforward as it is difficult to define what is relevant signal and noise. If we are only taking read noise into consideration, our images consistently have an SNR ~20. However, this does not seem like a relevant measurement. To estimate local Poisson noise that increases with signal intensity, we measured the standard deviation in a small homogenous cell membrane area and defined SNR as mean lysosome signal minus mean cell membrane background divided by this noise estimate. This analysis yields an SNR of ~3 that shows a small dependence on pHLARE expression level (Fig. S1D). Although the SNR is small, we believe it is enough to measure fluorescence in lysosome objects above the local background. It is also important to note that there are many sources of error in individual lysosome measurements (including movement between the two channel exposures) and that the reported pHlys per cell is the median of all these measurements to average noise and eliminate outliers.

In Fig. 2 (and Fig. S1A), the sfGFP channel in pH 5 nigericin looks dark because images are scaled to the same min/max intensity window to show the change in fluorescence, which at low pH is ~16% of the neutral pH value (Fig. S1B). To better illustrate that there is still plenty of signal at low pH to accurately measure intensities, we have modified the low pH sfGFP panel in Fig. S1A by splitting it on two halves with different intensity scales.

6. A large fraction of the manuscript is devoted to the torin and cancer cell data. The torin finding is not new and the differential pH of lysosomes of normal vs. transformed cells is inconsistent and debated in the literature. More importantly, no mechanism is proposed or investigated to account for any of these effects. These data should be omitted or at least greatly de-emphasized.

We respectfully disagree and believe data on lysosome pH with Torin-1 and cancer cells are highly significant. As we discuss in our manuscript, although effects of lysosome pH on mTORC activity (inside to outside signaling) have been reported, to our knowledge the effect of mTORC activity on lysosome pH has only been reported in one publication (Zhou et al., 2013 Cell Res. 23:508). This work suggested that pharmacologically inhibiting mTORC1 lowered lysosome pH; however, this was qualitatively determined by changes in LysoSensor fluorescence and absolute lysosome pH was not quantitatively measured. Our data confirm a decreased lysosome pH with inhibiting mTORC activity.

As Reviewer 1 correctly indicates, pHlys in cancer cells is controversial, which highlights the importance of our data quantitatively measuring pH in a broad range of cancer cells (we now include 11 distinct transformed cells in response to a request from the Monitoring Editor) from different tissue origins and with distinct mutational signatures. A few previous studies reported qualitative differences in cancer cells using non-ratiometric lysosome pH probes. Additionally, to our knowledge only one publication (Nilsson et al., 2010. Head Neck. 32:1185) compared cancer and tissue-matched non-transformed cells; however, this work used a very unconventional method to qualitatively and not quantitatively determine lysosome pH by loading cells with FITC-conjugated dextran and then separating cells based on fluorescence signal by using flow cytometry. Moreover, this latter work reported exclusively on head and neck cancer cells. In contrast, our study includes cancer cells from multiple tissue types, including breast, pancreatic, brain, lung, colon, and prostate. Hence, we
strongly believe the new data we include on many different types of cancer cells, comparisons with tissue-matched untransformed cells, and quantitative lysosome pH warrant the emphasis in our manuscript.

Other comments

1. The authors state that current reagents such as dextrans are limited because they are taken up by phagocytosis and are (therefore?) not exclusively localized in lysosomes. To my knowledge dextrans are taken up by endocytosis or macropinocytosis, but not by phagocytosis, which explains why they have been used in cells that are not phagocytic. Moreover, the dextrans can be restricted to lysosomes by using judicious pulse-chase protocols. In fact, such protocols ensure better localization to lysosomes than can be achieved by LAMP targeting, since LAMP is widely acknowledged to be present in late endosomes and, as the authors show, mistargets to the plasma membrane also. A more balanced discussion of these issues is necessary.

As requested, our revision includes edits as indicated by Reviewer 1, including dye uptake by endocytosis and micropinocytosis. We agree that while fluorescent dextran with endocytic uptake have more specific lysosome localization than fluorescent dyes such as LysoSensor that diffuse into lysosomes and have a high background signal in the cytoplasm. Also, as indicated by Reviewer 1, the very long (up to 24 h) pulse chase protocol for incorporating both dyes and dextran into lysosomes, in our view is a limitation of these reagents. Our revised introduction includes these points.

2. Oregon green 488 and 514 dextran conjugates can in fact be used for ratiometric imaging and do not need to be used in conjunction with another fluorophore. Oregon green 488 can be excited at both 490 nm and 440 nm wavelengths and Oregon green 514 can be excited at both 510 nm and 450 nm using a single emission wavelength for ratiometric pH imaging.

We agree that dextran conjugates are suitable for ratiometric imaging. However, the goal of our study is to generate a genetically encoded lysosomal pH biosensor that will allow for long-term expression in cell lines and in model organisms. These goals cannot be achieved by dextran conjugates. As noted in the manuscript, dextran conjugates are not exclusively localized to lysosomes because these reagents are incorporated by endocytosis and accumulate in multiple types of intracellular vesicles. Additionally, dye loading into cells requires hours; often 6-8 h for uptake and another 24 h for transport through endo-lysosomal trafficking (Nilsson et al., 2010; Wolfe et al, 2013; Johnson et al., 2016). For these reasons, we believe pHLARE is an improved reagent for measuring pHlys dynamics.

3. The authors note that lysosome size increases when using chloroquine to alkalinize whereas bafilomycin does not. While bafilomycin increases the pH by inhibiting the proton pump, there is literature suggesting that chloroquine impairs lysosome function and can induce lysosomal damage and even permeability. For example, it can directly inhibit palmitoyl-protein thioesterase 1 (PMID: 30442709). It is possible that chloroquine-induced lysosomal damage results in enlarged/swollen lysosomes. It is also unclear how stimulation of lysosome biosynthesis by TFEB would account for the size change. Why would the new lysosomes be any bigger?
Chloroquine-induced lysosomal swelling has been previously observed. Gallagher et al (2017) found incubation of cells with 25µM chloroquine increased the diameter of lysosome to 3-5 µm compared with 1-2 µm in control cells. This report found that lysosome swelling is dependent on the presence of glucose in the medium and is not seen with glucose starvation or treatment of cells with 2-deoxyglucose. The ability of chloroquine to increase pHlys, however, was independent of lysosomal swelling. Beyond findings this report, which we now add to our text, the precise molecular mechanism, including the role of PPT1 and TFEB, is currently unknown.

4. Why were cells treated with ammonia for 18 hrs, when the weak base alkalinizes in seconds?

As requested, we include new data on lysosome pH in RPE cells treated acutely (15 min) with NH₄Cl. These new data are included in Fig. S3. Acute effects of NH₄Cl, which we have measured extensively for pHi, require higher concentrations of ~ 30 mM, which is why we initially reported 18 h incubations with a lower (5 mM) concentration. As shown in Fig. S3, both conditions with NH₄Cl significantly increase pHlys in RPE cells.

5. Is Lysosensor in fact a dextran conjugate, as stated?

Reviewer 1 correctly questions a mistake in our manuscript. As indicated in ThermoFisher specifications LysoSensor™ dyes are acidotropic probes that appear to accumulate in acidic organelles as the result of protonation. We edited the text accordingly. This specification does indicate a limitation of LysoSensor targeting to acidic organelles being dependent on the acidic lumen of the organelle, which if compromised (in disease or for example with experimental use of bafilomycin and chloroquine) affects localization of the reagent.

6. The differential pH of normal cells and those of patients with neurodegenerative diseases is far from well-established and has been debated and disproven in some cases. A more balanced presentation of the field should be offered.

As requested, we revised text descriptions in the Introduction and Discussion on lysosome pH and neurodegeneration to be controversial, despite a currently accepted view that lysosome functions are dysregulated in neurodegenerative disorders.

Reviewer 2

Major

My first concern is the choice of cell type. Are the RPE cells pigmented? Could melanin affect the fluorescence signal? Even if the cells are not pigmented they might still contain melanosomes. Since melanosomes are LRO (lysosome related organelles), they contain several lysosomal proteins e.g. LAMP proteins. Could pHLARE in transfected cells be expressed in melanosomes as well? The pH of melanosomes increase during maturation. When studying the frequency of pH in single lysosomes (Figure 2D), there are actually "two peaks"; one at pH 4-4.5 and one at pH 5.75-6. Could they represent two different organelles - lysosomes and (immature) melanosomes? Separating melanosomes from lysosomes are
challenging but I recommend the authors to measure if melanin is present, as well as check the expression of melanosomal protein markers e.g., NKI/beteb (early melanosomes) and tyrosinase (late melanosomes).

We thank the reviewer for pointing out the possibility of caveats if RPE cells contain melanosomes. However, it is documented that clonal RPE cell lines do not contain pigment granules - both melanosomes and lipofuscin (Exp Eye Res. 2014;126:61; Scientific Reports. 2019;9:13761), and lack key enzymes in the melanogenesis pathway (Mol Vision. 2007;13:2066).

In the previous manuscript, we were intrigued by the bimodal distribution of individual lysosome measurements and speculated that there might be a biological reason or relevance to this. However, we did not discuss this because this set of six cells used for our initial development of the pHLARE analysis was admittedly rather small. For reasons also discussed below, we replaced this set of with a three times larger population of cells. We also now improved the analysis by calculating a frequency distribution for each cell and averaging these histograms instead of pooling all lysosome measurements together, which biased our previous analysis toward cells with more measurements. This new histogram (now Fig. 2B) is much tighter and no longer shows a bimodal distribution.

*Images showing the colocalization between sfGFP and mCherry is not convincing. In figure S1, overlay images seem to have vacuolar structures with only sfGFP (blue) and only with mCherry (magenta). The fluorophores are situated on either side of the lysosomal membrane - is there a 100% overlap? What is the maximum distance between two fluorophores that are presented as one pixel (i.e., colocalized in images)? Clear description of the optical limitation of the microscope, and the resolution of the images should be presented.*

*Figure 1B: Is mCherry specific for lysosomes? The image shows bright staining at the plasma membrane. The same comment for Figure 2A in which sfGFP seems to be present unspecific.*

The reviewer is correct that there is incomplete overlap between the different wavelength channels. These are images of live cells. As we can only acquire them sequentially, even at short timescales of <1s, lysosomes change slightly in shape and position. Imaging was done on a spinning disk confocal in which resolution is limited by standard optical diffraction. The measured lateral FWHM of the point spread function on our instruments is ~350 nm. The pixel size in images represents the camera pixel size of ~80 nm to appropriately sample the optical resolution but is not relevant to what can be theoretically resolved by the microscope. In larger lysosomes spinning disk confocal optical resolution can be sufficient to resolve the membrane, but in smaller lysosomes it is not. Thus, depending on size, there is a difference in lysosome appearance and at the resolution limit, mCherry, which resides on the outside may appear more membranous and sfGFP more luminal. As requested, we included a sentence about the resolution limits of the instrumentation used, which is also described in the methods section.

The reviewer is also correct that not all pHLARE localizes to lysosomes and a substantial fraction resides in the cell membrane. It is important to note that our pHlys analysis takes this local background into account and that this signal does not contribute to the measured
lysosome object fluorescence. We had discussed this in the previous version of the manuscript, but also in response to reviewer 1, have clarified this further in the main text. To properly quantify the amount of pHLARE fluorescence signal that is associated with lysosomes, we further modified and improved our co-localization analysis. We now use SiR Lysosome as a benchmark and measure pHLARE fluorescence in regions defined by SiR Lysosome. This new as well as our old object-based analysis of overlap show that pHLARE localizes to lysosomes at least as good or better than LysoTracker. Please also see our response to Reviewer #1’s first two comments.

*It is not clear to me how the authors calibrate each set of experiment. Is a new "standard curve" constructed for every experiment? In materials and method, pH 5.0 and 6.5 nigericin samples is mentioned. Is this the day-to-day calibration? To construct a standard curve at least three points should be analyzed to make sure that the slope is correct. What is the variation in fluorescence ratio between different experiments? What is the transfection degree in transiently transfected cells? Are all cells expressing equal level of the sensor? Several different cell lines are used and pH compared; how is the lysosomal pH calibrated between different cell lines? Is one calibration used for all different cell lines or is a separate standard curve constructed for each cell line? Please clarify.*

As requested, we more clearly describe in the Results and Methods sections the calibration protocol, which is done at the end of each determination. Hence, each imaging acquisition includes a separate nigericin calibration for that experiment and our analysis program uses a distinct calibration for each cell. One calibration is not used, neither for different cell lines nor for the same cell line over multiple cell preparations, different conditions or different cells within a population. As we and others have reported over many years for cytosolic pH calibrations, after establishing the linear range of the fluorescent ratio (which we do for pHLARE, Fig. 1D), we use a 2-point nigericin calibration at the end of each experiment that is within the linear range.

*It is not clear when the pH values are presented as median or as mean. Box and whisker plots are median but in Figure 1D pH is presented as means? Moreover, for example page 7, line 17-18 "The two calibration methods gave median pH values that were not significantly different (local background calibration pHlys = 4.95 \{plus minus\} 0.22 (mean and SD)......" Another example on page 10 " We used RPE cells stably expressing pHLARE and quantitative measurements to show that Torin-1, [.....]decreased pHlys from 5.22 \{plus minus\} 0.22 \{plus minus\}
0.10 in controls to 4.63 \{plus minus\}
0.09 \{mean \{plus minus\}
s.e.m., 4 cell preparations) (Fig. 4D)." Similar in Figure 5. Please, clarify how median and mean values are used.*

As discussed in the main text, to eliminate outliers due to measurement errors, we use the median value of all lysosome objects measured in one cell to determine a pHlys for that cell. All box plots show this one pHlys measurement for many cells. If pHlys values are reported in the text, they are mean +/- s.e.m. of pHlys of many cells. We clarified this in the text throughout. Only Fig. 1C (formerly D) is an exception and simply shows ratios of total cell fluorescence to illustrate the ratiometric dynamic range of pHLARE.
The authors argument that the pKa=4.5 of mCherry makes it insensitive to changes in cytosolic pH and therefore allows ratiometric analysis. However, during calibration with nigericin at pH 4.4 (slightly below pKa) the fluorescence of mCHerry seems to be affected (figure 2B). How is this affecting the standard curve? This needs to be considered and discussed.

Images in our original Fig. 2 were from early determinations during our development of pHLARE when we initially used a low pH calibration at around 4.5. In this case, mCherry fluorescence does indeed drop substantially. Once we identified this problem, low pH nigericin calibrations are now always at or above pH 5. In this case, the drop in mCherry fluorescence combined with potential photobleaching is less than 10% compared with neutral pH (Fig. S1C). Fig. 2 was now replaced with a newer and larger population of cells with the appropriate nigericin pH range, and we are discussing this limitation in the text. Note also that in Fig. 2 all images are scaled to the same intensity window also showing qualitatively very little difference in the mCherry channel at different pH values (in contrast to the sfGFP channel).

Transfections always affect the characteristics of the cell. In our hands, transfection with GFP-LAMP1 reduces the ability of lysosomes to move. In these experiments two fluorophores is fused to LAMP1. Is the locomotion of lysosomes affected? How is the endogenous protein levels of e.g., LAMP1 and LAMP 2 in transfected cells compared to wt? Some experiments to illustrate if the pHLARE transfected cells are functionally comparable to wt or if they differ, would be good to show.

As requested, we measured lysosome motility and found no significant difference between mean speed, maximum speed or displacement of lysosomes in pHLARE-RPE cells compared to wild-type RPE cells. These new data were added to Figure 3.

Figure 1E: An "unspecific band" is used as loading control. Why not use a proper housekeeping protein as loading control?

As requested, our revision includes a new immunoblot (Fig. 1E), with the membrane probed with RFP antibodies for pHLARE and GAPDH antibodies for a loading control.

Figure 3B: The image should be larger and a magnification bar added. Markings in the image presenting how the different areas are selected should be added (nucleus, perinuclear lysosomes, lysosomes in distal membrane protrusions).

As requested, our revised manuscript includes an enlarged image with the indicated areas in hatched lines in Figure 3B.

Most perinuclear lysosomes appear in clustered. Is it a risk that these are deselected and a bias introduced when only well separated lysosomes are analyzed?

Indeed, lysosomes are often close to each other and are frequently not separated in the segmentation. At this point, we have no good method to do this reliably. Thus, individual
measurements in cells represent technical replicates and should not be interpreted as individual lysosome pH values. We are discussing this now more clearly in the main text and for this reason also call segmented areas lysosome objects and not lysosomes. All lysosome objects are weighted equally. Thus, individual lysosomes and smaller clusters, which we however think yield more reliable pH value measurements, contribute more to the cell’s median pHlys value. To date, we have not been successful to consistently correct for this bias but may attempt to do so in future development of the analysis algorithm.

On page 8 the Figures are presented in random order: Fig 3A - Fig 5B and G - Fig 5E - Fig 5A - Fig 2D. Rewrite the text or rearrange the figure so that the results can be presented sequentially.

The order of figure presentation is not really random but does indeed “jump forward” to indicating data in Fig. 5 to limit repetition. Our previous Fig. 3 included data on non-transformed cells, as did Fig. 5. We believe maintaining data on non-transformed breast (MCF10) and pancreatic (HPDE) epithelial cells together with tissue-matched cancer cells is important for comparison. Also including data with these cells in Fig. 3 would be repetitive. With new data on more cell lines and new figures, as requested by the Managing Editor, the “jumping forward” is in part alleviated and in addressing the reviewer’s concern we edited the text description on non-transformed cells associated with Fig. 3 for improved clarity.

Figure S3: Why is cells pre-incubated during 18 h with NH4Cl? Most protocols use 30 min.

As requested, and as indicated in our response to Reviewer 1 (#4 above), we include new data on lysosome pH in RPE cells treated acutely (10 min) with NH₄Cl. These new data are included in Fig. S3.

Figure 4B: Concentration of chloroquine is higher than in most protocols - could such high concentration cause lysosomal membrane permeabilization? The increase in lysosome size shown in Figure 4A could then be interpreted as lysophagy of damaged lysosomes.

As indicated above in our response to Reviewer 1 questions on chloroquine, lysosome swelling is seen even at lower concentrations and is possibly related to glucose in the medium but has no effect on pHlys.

Figure 4D-E: I do think the analysis of mTOR effect of pH is interesting but the results must be accompanied with a more thorough analysis of the cellular effect of Torin-1 exposure. Is autophagy altered, is the size and localization of lysosomes changed?

As requested, our revision includes a representative image of RPE cells treated with Torin-1 (Figure 4D), which indicates no difference in the size or localization of lysosomes compared with control RPE cells (Figure 4A). Determining the mechanisms mediating the effect, including autophagy, are beyond the scope of this initial report on pHLARE. We do, however, show efficacy of Torin-1 in inhibiting mTORC activity with data on pS6K.

Figure 4F: The authors should prove by a proliferation marker that the cells are truly quiescent.
The term quiescent is commonly used for cells maintained in decreased or no FBS. To accommodate the reviewer’s concern, however, we revised the text and figure to indicate 10% vs 0.2% FBS. These data are now in Figure S3C.

Figure 5 F and 5G is not included in the manuscript.

With new data and figures, this has been corrected. Thank you for noting our previous mistake.

I think that the authors should streamline their manuscript. Now, the Results contain a lot of references; passages that is "introduction", several comparisons of previously determined lysosomal pH and explanations (could rather be part of the discussion). Then most of the discussion is concentrated on comparing lysosomal pH in different cancer cell lines and pure speculations of the impact for invasion and drug resistance with help of references. I think that the focus of the manuscript is lost. (If the correlation between invasion and lysosomal pH is such interesting, an invasion/migration experiment is quite easy to perform since all cell lines are available.) However, I think that pros and cons of the method should be in focus here and the discussion on impact of lysosomal pH in cancer cell lines reduced.

As requested, we attempted to streamline our revised manuscript. For the Introduction, to highlight the need for an alternative approach to measure lysosome pH, we do believe including limitations on currently used reagents is important but have attempted to streamline in our revision. For the Discussion, we edited to highlight the prime focus on pHLARE as a new and improved reagent for quantitative determinations of lysosome pH dynamics. As requested by the Managing Editor, however, we now include data using even more distinct cancer cell lines. This raises a quandary on de-emphasizing discussion on lysosome pH in cancer cells when 2 of our 6 main figures focus on this topic. Moreover, our previous as well as new data on cancer cells do indicate distinct differences that we believe are important to address with predictions on cell phenotype, including what is known about invasive properties of cells with lower compared with normal lysosome pH as well as metabolic profiles.

The supplement showing the step-by-step instruction for pH calculation is very good. However, it is 109 pages; several are blank, some only denote NaN and at the end several tables that are poorly explained. Please delete unnecessary material or label correctly.

The excel spreadsheet that serves as a template for pHlys calculation was erroneously added to the PDF in the original submission. Because the spreadsheet uses indirect indexing to properly identify fluorescence measurements, it is prefilled with formulas to row 1000 to ensure no fluorescence measurements are missed, and these formulas output NaN if there is no corresponding value. However, evidently this spreadsheet should not be appended to a supplement PDF.

The MBoC checklist demand that the method of cell line authentication and the frequency of testing for mycoplasma contamination is presented. This information is not in the manuscript.
As requested, our revised Methods section includes information on testing for mycoplasma (PCR Mycoplasma Detection Kit abm # G238, and on authentication (IDEXX BioAnalytics).

Minor

Figure 1B and C - the colors should be explained (compare figure 2B where sfGFP (green) and mCherry (magenta) is clarify). Colocalization presented as white should also be described

As requested, we modified the figures accordingly.

What are the small yellow markings in figure 3B and 5A? If of importance it should be enlarged and explained, if not important deleted.

The yellow markings indicated in the figures are near outliers, as calculated by the Analyzelt program. For clarity, the font size is enlarged in our revision.

The typesetting must be thoroughly checked; superscript/subscript should be used in all chemical formulas; prefix of Greek letters must be checked.

Figure 2: the letter E is lacking in the panel
Figure 4: the letter C I lacking in the panel
There are two supplements numbered S3 - one should be S4
Page 7, line 17: "We validated this analysis by comparing pHlys measurements in six RPE cells (Fig. 2D). Must be Fig 2E?"

Font size of the y-axis (pH) is often too small

As requested, errors were corrected, and font sizes were revised.
2nd Editorial Decision

RE: Manuscript #E20-06-0383R
TITLE: “pHLARE: A New Biosensor Reveals Decreased Lysosome pH in Cancer Cells”

Dear Dr. Barber:

Your revised manuscript has now been reviewed by the two original reviewers and as you can see from their comments, they are satisfied with your revision. Thus the manuscript can be accepted for publication pending the correction of the few typos/mistakes listed by reviewer #2.

Sincerely,
Marja Jaattela
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Barber,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

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Reviewer #1 (Remarks to the Author):
The authors have addressed all my original comments and requests

Reviewer #2 (Remarks to the Author):

The revised version of the manuscript by Webb et al. is greatly improved. Comments have been addressed, the presentation of results are improved and central issues have been commented upon.

Minor comment:
Page 8: One sentence is repeated twice; see line 7 and 16 "Although we typically..." Delete.
Page 18: first line last word should be exocytic, not endocytic.
November 10, 2020

We are submitting a revised manuscript #E20-06-0383R entitled *pHLARE: A New Biosensor Reveals Decreased Lysosome pH in Cancer Cells*. Our revision includes the two text corrections requested by Reviewer 2.

Sincerely,

Diane L. Barber, PhD
Professor and Chair
Leland A. and Gladys K. Barber Endowed Chair in Dentistry
Department of Cell and Tissue Biology
RE: Manuscript #E20-06-0383RR
TITLE: "pHLARE: A New Biosensor Reveals Decreased Lysosome pH in Cancer Cells"

Dear Dr. Barber:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Marja Jaattela
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Barber:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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