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## **A novel family of fluorescent hypoxia sensors reveal strong heterogeneity in tumor hypoxia at the cellular level**

Raghu Erapaneedi, Vsevolod Belousov, Michael Schäfers and Friedemann Kiefer

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### **Review timeline:**

Submission date:	17 August 2015
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Editor: David del Alamo

### **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.)

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1st Editorial Decision

16 September 2015

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Thank you for the submission of your manuscript entitled "A novel family of fluorescent hypoxia sensors reveal strong heterogeneity in tumor hypoxia at the cellular level" and for your patience during the review process. We have now received the reports from the referees, which I copy below.

As you can see from their comments, referees #1 and #2 are very supportive of your manuscript and recommend only relatively minor modifications. Referee #3 is however less enthusiastic. I have discussed your case with Karin Dumstrei and we both believe that his/her concerns may be satisfied with a better explanation of the advantages of your technique over previous hypoxia reporters. Other concerns, particularly about the depth of functional or physiological insight should be disregarded as your paper was presented to the referees as a resource, and as such should be evaluated as a tool for the community

I believe the referee concerns are rather straightforward, but in any case, please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems.

Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

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REFEREE REPORTS

Referee #1:

Erapaneedi et al. report "A novel family of fluorescent hypoxia sensors reveal strong heterogeneity in tumor hypoxia at the cellular level". Fluorescent reporters of cellular hypoxia based on GFP or RFP are limited by a requirement for oxygen for efficient maturation. UnaG is a fluorescent reporter that does not require oxygen. By driving oxygen-dependent UnaG expression through the use of HIF responsive elements, while modulating its half life upon fusion with well characterized constitutive and oxygen-responsive degrons, the authors demonstrate that such constructs are effective as *in vitro* and *in vivo* reporters of hypoxia. By coupling these reporters with oxygen-dependent fusion proteins, the authors are able to identify differences in dynamic hypoxia amongst individual cells *in vivo*. Such constructs would appear to be useful reagents that can be easily adopted by the hypoxia community to address interesting questions in ways not accessible with current techniques.

Minor questions:

- 1) In Fig 3e, the lower region of hypoxia bounded by the red line would appear to contain significant mCherry signal in contrast to other hypoxic regions shown here. Why is this?
- 2) In Fig 4b, why does there appear to be much more cellular heterogeneity in the mOrange signal when compared to the UnaG signal of the same cells (ie in the 20 hr normoxia panel)?
- 3) The authors claim in Fig.5d and 5e that orange spots at the edge of hypoxic clusters correlated with apoptotic fate, though the evidence for this seems quite descriptive. Perhaps the authors could adopt a more quantitative criteria in support of this observation.
- 4) The prevalence of the signal will depend not only on the half-life of the protein but also the half life of the mRNA that accumulates under hypoxic conditions which can continue to be translated even upon a shift to normoxia. The authors might comment on the mRNA half-life of these constructs and whether manipulation of mRNA half-life might further extend the dynamics of hypoxia/reoxygenation that might be observed with these constructs.

Referee #2:

The manuscript by Erapaneedi describes novel biosensors for the detection of hypoxia *in vivo* and *in vitro*. This will add valuable tools to the Cell Biology and Cancer Biology toolboxes. The experiments evaluating the suitability of the sensors are competently executed and the paper deserves publication after some revision as outlined below:

Major issues:

Referencing has to be improved. Such the statements on HIF (Introduction, p. 1, line 4 from bottom to p.2, line 3) have to be referenced.

Results p. 1, line 6 from bottom: reference 16 should be included after "bilirubin". The following statement needs either experimental proof (i.e. determination of serum bilirubin concentration and addition of the same concentration of bilirubin instead of serum) or some qualification.

Fig. 2b is not consistent with the rest of the paper, showing a clear stimulation of GFP fluorescence by hypoxia. Is it possible that the colour codes for hypoxia and CoCl<sub>2</sub> have been mixed up?

Discussion: The first sentence of the fourth paragraph needs some qualification.

Discussion, p. 1, line 10 from bottom: The sentence beginning with "Our observations" does not make sense in the present form: No observations related to apoptosis are reported in the manuscript and the references are not publications from the authors.

Minor issues:

"*in vivo*" and "*in vitro*" should be italicised throughout the manuscript.

Introduction, p. 1, line 17 from bottom: delete "For".

Introduction, p. 1, line 10 from bottom: comma after translocator.

Results, p. 4, line for: replace "resided" by "decided"; "excite", not "exite".

Figure legend expanded figure 1a: replace "molecular weight" by "molecular mass"

Referee #3:

In this manuscript, Erapanedi et al. develop a panel of fluorescent reporter genes designed to provide information on the oxygenation status of cells into which they have been transfected. These genes utilize a recently reported fluorescent protein, UnaG, which unlike other fluorescent proteins such as GFP and RFP does not require post-translational modification by oxygen to produce an active fluorophore. They first develop and validate a reporter that is transcribed under the control of HIF-1 using a promoter containing hypoxia response elements (HREs) and whose protein lifetime is limited through inclusion of a PEST sequence, and demonstrate its induction and activity under hypoxic conditions relative to an HRE-GFP reporter. A range of HRE-driven reporters including oxygen degradation domains (ODDs) are then engineered and similarly evaluated. These reporters are applied in a tumor model *in vivo* and compared with HIF-1 stabilization. Finally, an HRE reporter encoding a fusion of UnaG and the oxygen-dependent fluorophore mOrange was created to report on areas of reoxygenation, and was utilized *in vivo* to investigate the spatial and temporal characteristics of tumor cell oxygenation.

The use of the UnaG fluorescent protein to create a reporter gene for hypoxia is novel, and overcomes limitations of previous fluorescent and luminescent oxygen-dependent constructs to accurately report on areas of poor oxygenation. The authors have rigorously validated the performance of these reporters *in vitro*. However, the oxygen dependence of reporter genes and its influence on hypoxia-specific reporters is well established (Cecic et al., *Mol Imaging* 2007) and a number of manuscripts have investigated this area as well as designed and characterized these types of HRE- and ODD-mediated reporters (Harada et al., *Biochem Biophys Res Comm* 2007). Therefore it is not apparent what real benefit is gained from the current reporters, which utilize similar designs but incorporate UnaG.

Moreover, the studies presented in this manuscript applying the reporters are observational and do not provide any useful new insights into the spatial or temporal distributions or significance of tumoral hypoxia. Importantly, the authors' conclusion that the presence of reoxygenated cells near blood vessels is evidence of cell migration ignores the possibility of transient vessel opening and closing and the resulting acute hypoxia, which is well described in the radiobiological literature (Jansen et al., *Int J Radiat Oncol Biol Phys* 2002). Even given the heterogeneity between adjacent cells shown in Figure 5b, it seems unlikely that a cell would migrate the distance from a blood vessel to a hypoxic region in less than the time that the reporter would turn over. More thorough intravital studies directly documenting cell migration are needed to properly evaluate this claim.

Specific Issues:

Figure 2d: The labeling of the columns is unclear, it is not apparent how these cells were treated with hypoxia and normoxia. It is recommended that a timeline be placed across the top to make this more clear.

Figure 3f: The color scale applied in this figure is difficult to interpret, it would be helpful to display the dUnaG and Qdot channels as single colors rather than a colorwash.

Figure 4c: The images do not appear to correspond to the intensities plotted in panel 4b. Specifically, the intensity of mOrange at 20 hours and 72 hours appears equivalent in 4b, but the images shown in 4c show much lower intensity at 20 hours than at 72. Similarly, the UnaG fluorescence at 58 hours is significantly higher than at 20 hours in 4b, but the images look equivalent.

Figure 5e: The association between reoxygenated cells and apoptosis should be established through direct measures of apoptosis such as TUNEL and/or caspase staining.

We thank the referee's for their thoughtful comments, which have helped us to correct errors and improve clarity and readability of the manuscript.

Response to the comments of Referee #1:

*Erapaneedi et al. report "A novel family of fluorescent hypoxia sensors reveal strong heterogeneity in tumor hypoxia at the cellular level". Fluorescent reporters of cellular hypoxia based on GFP or RFP are limited by a requirement for oxygen for efficient maturation. UnaG is a fluorescent reporter that does not require oxygen. By driving oxygen-dependent UnaG expression through the use of HIF responsive elements, while modulating its half life upon fusion with well characterized constitutive and oxygen-responsive degrons, the authors demonstrate that such constructs are effective as in vitro and in vivo reporters of hypoxia. By coupling these reporters with oxygen-dependent fusion proteins, the authors are able to identify differences in dynamic hypoxia amongst individual cells in vivo. Such constructs would appear to be useful reagents that can be easily adopted by the hypoxia community to address interesting questions in ways not accessible with current techniques.*

*Minor questions:*

*1) In Fig 3e, the lower region of hypoxia bounded by the red line would appear to contain significant mCherry signal in contrast to other hypoxic regions shown here. Why is this?*

We thank referee #1 for this positive and constructive criticism. There is a noticeable difference in the mCherry signal in the two encircled areas in Fig. 3e, that we have not alluded to in the manuscript. Figure 3e is an *intra-vital* image taken from a mouse implanted with a cranial window using multiphoton microscopy representing a 60µm tissue stack. Transplanted Gli36 tumor cells express a non-destabilized version of mCherry driven from the CMV promoter along with HRE-dUnaG. Therefore mCherry is expressed constitutively, while UnaG is expressed under hypoxia and the protein half-life of mCherry should be significantly longer than the destabilized UnaG. The noted difference could result from recent temporal changes in oxygen supply or different times the tumor cells have been exposed to hypoxic conditions. While we cannot rule out these effects, we favour an explanation that is more based on the geometry of the tumor within the tissue cube investigated. As shown in Fig. 3f both hypoxic islands are located towards the top of the imaged tissue (as indicated by the predominance of purple, red and yellow green colors). The two large, perfused vessels at lower edge of Fig. 3e (Qdots, depicted in turquoise) are located at the bottom of the imaged tissue cube (indicated by the dark blue and azure depth coding in Fig. 3f). This geometry will cause two effects. First, the bounded hypoxic region located top left, will experience a larger distance to the nearest perfused vessel compared to the bounded hypoxic region located bottom right. Second, the very same will even more so apply to the tumor cells underneath the hypoxic cap. Taken together this suggests that the mCherry signal originates from tumor cells that are positioned at the bottom of the imaged tissue volume and are flanked by a large perfused vessel, which would provide them with sufficient oxygen tension to upkeep a residual amount of mCherry signal. We consider it extremely exiting, that temporal as well as spatial influences on the developing hypoxic state within the developing tumor have now become accessible to analysis by optical imaging using our model system. However, a precise description will necessitate extensive intravital microscopy, which is presently in the center of a novel study. We have now discussed this issue in the main text on page 8 in the 2<sup>nd</sup> paragraph.

*2) In Fig 4b, why does there appear to be much more cellular heterogeneity in the mOrange signal when compared to the UnaG signal of the same cells (ie in the 20 hr normoxia panel)?*

This question of the reviewer refers to Fig. 4 and here in particular to Fig. 4c. Being alerted by the reviewer's comment to this seeming discrepancy, we noted that in particular the pseudo coloring of the original data using an orange hue causes in print, but also on the screen an apparent selective loss of the cells expressing the lowest mOrange levels. We have therefore decided to apply a grey scale for the visualization of this experiment. In this form of presentation different expression levels are far better represented and heterogeneity within mOrange cells is no longer visible. In addition under conditions of reoxygenation, i.e. maturation of mOrange, the strongest UnaG expressing cells score equally positive for mOrange.

We should also like to point out, that the images in Fig. 4c are directly taken from the accompanying movie (movie2) and the quantitation in Fig. 4b is solely based on these individual frames. As

detailed in the methods section, to avoid photo bleaching over the long imaging periods, the excitation laser energy was kept to the minimum allowing detection of a above background signal.

*3) The authors claim in Fig.5d and 5e that orange spots at the edge of hypoxic clusters correlated with apoptotic fate, though the evidence for this seems quite descriptive. Perhaps the authors could adopt a more quantitative criteria in support of this observation.*

Here, we fully concur with the notion of referee #1, because also referees #2 and #3 addressed the issue of the orange spots at the edge of hypoxic clusters, we performed TUNEL staining on brain sections to corroborate our notion that these spots were associated with high apoptotic activity. Indeed, we found that a high proportion of the re-oxygenated cells stained TUNEL positive, indicating an increased propensity to undergo apoptotic cell death. We included these results now in the manuscript as Fig. 5e.

*4) The prevalence of the signal will depend not only on the half-life of the protein but also the half life of the mRNA that accumulates under hypoxic conditions which can continue to be translated even upon a shift to normoxia. The authors might comment on the mRNA half-life of these constructs and whether manipulation of mRNA half-life might further extend the dynamics of hypoxia/reoxygenation that might be observed with these constructs.*

Here referee #1 raises an interesting issue, mRNA stability, which we have so far not actively addressed. In all our hypoxia constructs, the fluorescent proteins are expressed under the same hypoxia-inducible promoter. Since we used stably transfected cell bulks for all our experiments, effects on transcription depending on the sensor sequences integrating into transcriptionally active or inactive zones should have been minimized. In first approximation, we therefore presume that mRNA levels for all sensor constructs are similar, however, we cannot exclude changes in mRNA stability due to the different sequences of the sensor constructs.

For the purpose of this study, we found the modulation of protein stability by various constitutive and oxygen-responsive degrons fully sufficient, in particular, as reduced stability also results in reduced overall signal intensity i.e. brightness. In future studies, modulation of sensor mRNA half life could present an interesting approach to enhance the dynamics of the UnoHR sensors at protein level far beyond the scope of this work.

*Referee #2:*

*The manuscript by Erapaneedi et al. describes novel biosensors for the detection of hypoxia in vivo and in vitro. This will add valuable tools to the Cell Biology and Cancer Biology toolboxes. The experiments evaluating the suitability of the sensors are competently executed and the paper deserves publication after some revision as outlined below:*

*Major issues:*

*Referencing has to be improved. Such the statements on HIF (Introduction, p. 1, line 4 from bottom to p.2, line 3) have to be referenced.*

We thank referee #2 for the insightful evaluation and valuable comments. We have now improved referencing in the entire introduction section, and in particular included additional references on the items HIF, FIH, HREs and HIF target genes.

*Results p. 1, line 6 from bottom: reference 16 should be included after "bilirubin". The following statement needs either experimental proof (ie., determination of serum bilirubin concentration and addition of the same concentration of bilirubin instead of serum) or some qualification.*

We have now included reference 16 in the text and in the subsequent text elaborated on the concentration of unconjugated Bilirubin in our serum supplied for tissue culture, which is 2 µg/ml. This allows us to estimate that for the cell lines described in our study approx. provision of 200 nmol/l bilirubin will result in saturated UnaG fluorescence.

*Fig. 2b is not consistent with the rest of the paper, showing a clear stimulation of GFP fluorescence by hypoxia. Is it possible that the colour codes for hypoxia and CoCl<sub>2</sub> have been mixed up?*

We thank the reviewer for pointing out this error. The color coding of this figure was indeed mixed up, we corrected this mistake in the revised manuscript.

*Discussion: The first sentence of the fourth paragraph needs some qualification.*  
We have corrected this oversight and introduced appropriate references.

*Discussion, p. 1, line 10 ff from bottom: The sentence beginning with "Our observations" does not make sense in the present form: No observations related to apoptosis are reported in the manuscript and the references are not publications from the authors.*

This part of the manuscript has been thoroughly rephrased and we now refer to the novel data obtained by TUNEL staining, which support our previous argument on apoptosis. The corresponding results are presented in Fig. 5e.

*Minor issues:*

*"in vivo" and "in vitro" should be italicised throughout the manuscript.*

*Introduction, p. 1, line 17 from bottom: delete "For".*

*Introduction, p. 1, line 10 from bottom: comma after translocator.*

*Results, p. 4, line for: replace "resided" by "decided"; "excite", not "exite".*

*Figure legend expanded figure 1a: replace "molecular weight" by "molecular mass"*

We thank referee #2 for the careful correction, we have corrected all issues.

*Referee #3:*

*In this manuscript, Erapanedi et al. develop a panel of fluorescent reporter genes designed to provide information on the oxygenation status of cells into which they have been transfected. These genes utilize a recently reported fluorescent protein, UnaG, which unlike other fluorescent proteins such as GFP and RFP does not require post-translational modification by oxygen to produce an active fluorophore. They first develop and validate a reporter that is transcribed under the control of HIF-1 using a promoter containing hypoxia response elements (HREs) and whose protein lifetime is limited through inclusion of a PEST sequence, and demonstrate its induction and activity under hypoxic conditions relative to an HRE-GFP reporter. A range of HRE-driven reporters including oxygen degradation domains (ODDs) are then engineered and similarly evaluated. These reporters are applied in a tumor model in vivo and compared with HIF-1 stabilization. Finally, an HRE reporter encoding a fusion of UnaG and the oxygen-dependent fluorophore mOrange was created to report on areas of reoxygenation, and was utilized in vivo to investigate the spatial and temporal characteristics of tumor cell oxygenation.*

*The use of the UnaG fluorescent protein to create a reporter gene for hypoxia is novel, and overcomes limitations of previous fluorescent and luminescent oxygen-dependent constructs to accurately report on areas of poor oxygenation. The authors have rigorously validated the performance of these reporters in vitro. However, the oxygen dependence of reporter genes and its influence on hypoxia-specific reporters is well established (Cecic et al., Mol Imaging 2007) and a number of manuscripts have investigated this area as well as designed and characterized these types of HRE- and ODD-mediated reporters (Harada et al., Biochem Biophys Res Comm 2007). Therefore it is not apparent what real benefit is gained from the current reporters, which utilize similar designs but incorporate UnaG.*

We fully concur with referee #3 on the notion that the oxygen-dependence of reporter genes and hypoxia-specific reporters are well established and have previously been characterized. In line with the referee's notion and to emphasize the present state of the art, we have included the suggested additional references into the manuscript. However most importantly, the well-known oxygen-sensitivity of the established reporter genes led to the dilemma that despite excellent reporter constructs based on hypoxia-specific genetic and protein control elements until now the positive identification of hypoxia by optical microscopy with cellular resolution was impossible. This point is excellently highlighted in the reference by Cecic (Cecic et al., Mol Imaging 2007) mentioned by the referee, which states that for optical imaging of hypoxia only "constitutively expressed reporter

constructs could be used to image regions of acute hypoxia by looking for signal decreases (negative contrast).”

Due to the possibility of malfunction, the absence or disappearance of a reporter will always be more ambiguous compared to a positive i.e. newly appearing reporter. Therefore, we are firmly convinced that the use of UnaG not only (and here we quote referee #3) “overcomes limitations of previous fluorescent and luminescent oxygen-dependent constructs to accurately report on areas of poor oxygenation” but indeed provides a completely new quality with tremendous benefit for optical hypoxia imaging. Importantly, this reporter system is entirely genetically encoded and does not rely on the administration of additional reagents, which would necessarily depend on perfusion / diffusion. In addition, it can be visualized without technical modification with existing fluorescence microscopes including *intravital* multiphoton setups.

*Moreover, the studies presented in this manuscript applying the reporters are observational and do not provide any useful new insights into the spatial or temporal distributions or significance of tumoral hypoxia. Importantly, the authors' conclusion that the presence of reoxygenated cells near blood vessels is evidence of cell migration ignores the possibility of transient vessel opening and closing and the resulting acute hypoxia, which is well described in the radiobiological literature (Jansen et al., Int J Radiat Oncol Biol Phys 2002).*

We thank reviewer #3 for this valuable comment and have now mentioned the possibility of transient tumor vessel occlusion. However, we would like to emphasize that the cited reference by Jansen et al. considers transient vessel occlusion as **one** possible explanation for discrepancies between the diffusion limited fraction (DLF) and pimonidazole staining in human tumor samples. In such tumors, the frequency of IdUrd-negative vessels, which was used as a perfusion marker, was increased (p=0.05) leading the authors to conclude that (quote) “although the assumption is reasonable that IdUrd-negative regions are acutely hypoxic, further information and validation are needed.” Indeed, our reoxygenation reporter will now allow the in depth investigation of these questions, providing insights far beyond those that can be derived from tissue sections.

*Even given the heterogeneity between adjacent cells shown in Figure 5b, it seems unlikely that a cell would migrate the distance from a blood vessel to a hypoxic region in less than the time that the reporter would turn over. More thorough intravital studies directly documenting cell migration are needed to properly evaluate this claim.*

While transient vessel closing could explain the appearance of reoxygenated cells near blood vessels, it would not explain the heterogeneity of individual cells. At least in our *in vitro* experiments, we could demonstrate synchronous response of our reoxygenation reporter of several hypoxia / reoxygenation cycles. Friedl et al. (Microsc.Res.Tech. (1998) 43, p369-378) determined the speed of slowly migrating large polarized cells in 3D matrices to be around 0.5µm/min. Given the maximum mOrange fluorescence in our reoxygenation reporter being observed around 4 to 6 hours after reoxygenation (Fig. 4b) this would amount to a migration distance of 120 to 180 µm, which we believe would fit well with the distances observed in Figure 5b and c. We now discuss this in the main text of the manuscript on page 12, paragraph 2. Again, we fully agree with referee #3 on the need for significantly more and detailed intravital imaging studies, which however are beyond the emphasis of this study that provides a first description of this new reporter system and demonstrates its principal *in vivo* applicability.

#### *Specific Issues:*

*Figure 2d: The labeling of the columns is unclear, it is not apparent how these cells were treated with hypoxia and normoxia. It is recommended that a time line be placed across the top to make this more clear.*

Fig. 2d provides selected frames from the experiment depicted in 2b. To make this more clear, we have indicated the sampling time by red arrows in Fig. 2b and also elaborated on the treatment conditions in the figure legend and methods section. Similarly, we placed the time line (legend) on top of the panels of Fig. 2c and elaborated in figure legend and methods as well.

*Figure 3f: The color scale applied in this figure is difficult to interpret, it would be helpful to display the dUnaG and Qdot channels as single colors rather than a colorwash.*

We conclude from the reviewer's important comment, that we have not been detailed enough in explaining this figure. Fig. 3f provides a z-dimension depth color coding of the UnaG and Qdot channels shown in Fig. 3e. Because Fig. 3e is a maximum intensity z-stack projection covering 60µm, valuable information on the distribution of the signals in the z-dimension is lost. To provide this depth information to the reader, we have included the additional Fig. 3f. In this representation, identical colors in the Qdot and UnaG channel represent the same z-position / depth. As the referee has pointed out correctly, this form of display tends to become rapidly overloaded upon inclusion of too much signal. We have therefore limited the z-dimension to a substack of 60µm of the originally acquired data.

*Figure 4c: The images do not appear to correspond to the intensities plotted in panel 4b. Specifically, the intensity of mOrange at 20 hours and 72 hours appears equivalent in 4b, but the images shown in 4c show much lower intensity at 20 hours than at 72.*

The long term imaging experiment shown in this figure was running for 72 hours. To avoid cell damage and photobleaching, we minimized the excitation laser intensity as much as possible. Therefore we contend that the visual representation is not trivial as the grey levels that differentiate images at various time points are low, due to the low excitation intensities. As already pointed out in our response to referee #1, we realized that the use of green and orange hues in our previous version was suboptimal in this setting and we have therefore now switched the Fig. to a greyscale representation, which significantly gives a better representation of the true intensity values. However, we again emphasize that all images that are shown in Fig. 4c were taken from the accompanying movie and the graph in Fig. 2c was quantified from these primary data. We should like to stress the point that in Fig. 4b the intensity of mOrange at 72 hours is about thrice the intensity at 20 hours, which is now well represented in Fig. 4c.

*Similarly, the UnaG fluorescence at 58 hours is significantly higher than at 20 hours in 4b, but the images look equivalent.*

In addition to all points raised with regards to the mOrange signal, the UnaG fluorescence at 58hrs is indeed only marginally higher as compared to 20hrs, which we feel, is now well represented by Fig. 4c. The higher signal increase in the second reoxygenation cycle in comparison to the first one is due to the proliferation of cells in the live cell culture; which we have mentioned on page 9, 1<sup>st</sup> paragraph in the main text.

*Figure 5e: The association between reoxygenated cells and apoptosis should be established through direct measures of apoptosis such as TUNEL and/or caspase staining.*

We fully concur with the referee and have performed TUNEL stainings on tissue sections, which indeed confirmed that a large fraction of the cells in those areas, where we had suspected an increased apoptosis rate based on nuclear fragmentation, stained positive for TUNEL. These data are now included in Figure 5.

Thank you for the submission of your revised manuscript to The EMBO Journal. Given the original minor concerns from the referees and the revisions provided, I am writing with an 'accept in principle' decision, which means that I will be happy to formally accept your manuscript for publication once a few more issues have been addressed.

Browsing through the manuscript, I have found a very minor problem that you need to correct: Expanded View figures 1 and 4 lack scale bars. These should be added to the immunofluorescence images and explained in the corresponding legend.



Thank you very much for your patience. I am looking forward to seeing the final version of your manuscript. Congratulations in advance for a successful publication.

2nd Revision - authors' response

23 October 2015

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We are very pleased about the positive response of the reviewers and appreciate your and the reviewers notion that the emphasis of our manuscript is on the description of a set of generally applicable tools for the hypoxia research community, which we support by several *in vitro* and *in vivo* experiments, including *ex vivo* histology and intravital multiphoton microscopy.

We are grateful for the reviewers' constructive criticisms, which we have all addressed in our revised manuscript and pointed out in our point by point response to the reviewers. One issue that was raised by all three reviewers referred to our notion of a high rate of apoptosis observed in the cells identified as recently reoxygenated. To provide further experimental support, we have now included TUNEL staining on tumor sections and demonstrate a clear match between reoxygenated and TUNEL-positive tumor cells in the main Fig. 5e.

#### EDITOR COMMENT

Expanded View figures 1 and 4 lack scale bars. These should be added to the immunofluorescence images and explained in the corresponding legend.

*Scale bars have now been included for Figures EV1 and EV4 and noted in the accompanying legends.*