

Interactive comment on “Determination of the metabolically active fraction of benthic foraminifera by means of Fluorescent in situ Hybridization (FISH)” by C. Borrelli et al.

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Anonymous Referee #2

The topic of this paper should be of interest to a number of foraminiferal ecologists, which are probably not a major portion of the Biogeosciences audience. In that regard, it is not obvious that this journal is the best possible journal for this contribution. We do not agree with this comment as several scientists have chosen this journal to publish studies about the biological, molecular, biochemical and ecological features of benthic foraminifera (Barras et al., 2010; de Nooijer et al., 2009; Filipsson et al., 2010), including aspects of biodiversity and ecology. In any case we think that the decision is in the hands of the editor.

As for the approach, the rationale is adequate: there are no perfect methods to differentiate live

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and dead foraminifera. This manuscript, however, oversimplifies this new method and does not fully explain the negative aspects of such an approach. Below are listed a number of issues that must be addressed by these authors before this paper can be published in any reputable journal. Please note that the majority of this review was written prior to the posting of Dr. de Nooijer's comments. In some cases the following points reiterate those made by de Nooijer and, in general, I agree with his assessment.

We completely agree with the Referee, in the sense that there are no perfect methods for distinguishing live and dead foraminifera (please, see also the letter to the editor as introduction to de Nooijer's comment answers), but we are convinced that the method proposed here is better when compared with other existing methods. Science proceeds by further implementation and approximation and we hope to be able to provide a contribution to this process in foraminifera studies. In any case, in order to accomplish the requests made by ref#2, in the amended version of the manuscript, we will explain the advantages and limitations of the FISH method.

1-> How did the authors distinguish between the dim fluorescence in (1) "washed shells" (page 7483 line 20, Figure 2H), (2) specimens exposed to nonsense probes (Figure 3B), and (3) starved (but living) individuals (Figure 5)? This is a major problem with this approach. Figure 2H ("washed shell") shows that FISH does not occur on dead specimens or, as in this specific case, specimens which have been washed from after a treatment with NaClO. Instead, figure 3B (specimen treated with a "non-sense" probe, which not which has no known rRNA target and has been included in the probe evaluation to ensure nonspecific incorporation of the probe into the sample does not occur) displays that the FISH protocol is adequate and do not generate false fluorescence signals.

As far as the level of background fluorescence is concerned, several authors have shown that the FISH technique may result in a very low-intensity background signal even from non-stained cells (see for instance the Figure 3 in the paper on marine protists by Stoeck, Fowle and Epstein 2003 Appl Environ Microb). However, this signal is always sufficiently low and not sufficient for the operator to score a cell as "live" or "active".

Conversely, from our pictures, it is clearly evident that the signal from the live but "starved" individual in Figure 5 is much more intense than the background signal from the washed shell (figure 2H) and from the non-sense probe (3B).

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In the amended ms, the quality of the photos will be improved and we will possibly add a scale of fluorescence. In this sense, it will become clearer that the picture of “starved” cell (shown in Figure 5) emits a higher fluorescence signal than the two other cited. The fluorescence signal in Figure 5 assumed to be to the minimal fluorescence intensity for scoring a cell as “live” (even if characterized by a low activity when compared with the other shown in the same figure).

2-> The contribution relies on extremely low numbers where there is no statistical power whatsoever. In addition, for example, the efficacy of this approach was only 50% for *Eggerella* (page 7485 line 5). It is impossible to say what the efficacy of the method was for the other species given the text is not possible to understand (see also next point). Despite the limited numbers of species and individuals, our results are clear in showing that FISH analysis is appropriate to detect the active and live components of the foraminiferal fauna (i.e. within the order Foraminifera). However, in order to accomplish ref#2 requests, in the amended version of the manuscript, we will add results from additional analyses.

3-> Some sections of the text are un-interpretable (e.g., page 7484, Line 15-22; Table 3). Of these 19 *Ammonia*, how many were living? Although the text states that 17 emitted fluorescence, the text does not say if they were live or dead as determined by optical means.

Ref#2 is right, in the amended version of the manuscript, we will reshape all the Tables.

4-> The same question arises for the other 7 that were incubated with S17. Thus, it is impossible to know what the efficacy of this method was. Also, Table 1 appears to show that the FISH approach overestimates the proportion of living specimens (but, frankly, the table is uninterpretable).

We will clarify this aspect and discuss the limits and advantages of the FISH method as an alternative approach to discern live/dead organisms. To our opinion the point is that FISH DOES NOT overestimate, rather provides indication of the living cells even when these do not move or have evident pseudopodia under the light microscopy.

5-> What is the bulge in the upper right in Figure 4B? It does not appear in the “optical” image (A), and why is there another fluorescent object to the right of the foraminifer? Please clarify why these label with the FISH probe.

The referee is right. The two pictures did not refer to the same individual. We have the right

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pictures to replace these ones.

6-> Specimens were air dried (page 7481, line 20), so why does the fluorescent signal appear to include the entire volume of the shell (see Figures 4-5)? When dried, foraminiferal cytoplasm will not fill all chambers of their shells (cytoplasm is mostly water, and thus when dried, it will only fill a small fraction of the shell). Along this line of argument, it is extremely peculiar that the fluorescence was homogeneous in the protoplasm given these specimens were dried (page 7485, line 9-15). Imagine a grape versus a raisin. It is impossible that dried cytoplasm would have remained homogeneously distributed in dried shells. Thus, the fluorescence cannot represent foraminiferal cytoplasm.

We do not agree as the “air drying” step is an integral part of the FISH protocol for prokaryotes and do not affect the subsequent hybridization procedure (see Pernthaler, Glöckner, Schönhuber and Amann “Fluorescence in situ hybridization with rRNA-targeted oligonucleotide probes” Methods in Microbiology 2001). Specimens were first fixed with PBS and ethanol and, after that, the solution was removed by exposure under to LAF (Laminar Air Flow). Conversely, if ethanol is not removed, it will negatively affect the FISH results, as traces of ethanol can prevent probe hybridization. In addition: during FISH hybridization, each probe molecule will theoretically hybridize with a molecule of rRNA. Consequently, each cell will show a more or less homogenous fluorescence signal, depending of the concentration and localization of ribosomes inside the cells. It is expected that a natural amplification of the fluorescent signal, result of the large numbers of ribosomes in active cell, will occur.

Plus, to test false positives of recently dead specimens (page 7482 line 17), why were specimens dried for an hour when was a step used in the actual FISH protocol (page 7481 line20)?

This part was not clearly presented in the original version. The protocol at pag. 7482 refers to the “Specimen washing” experiment, which was performed with the only aim of testing the possibility that the probe could hybridize with the inner organic layer of the shell, the ectoplasm or other organic residues of recently-dead organisms.

Non-specific staining of FISH probe can sometimes occur under non-stringent laboratory condition, thus potentially providing false-positive results for dead organisms (Hugenholtz, Tyson Blackall, in “Methods in Molecular Biology”). Conversely, the “air drying” step is an integral part of FISH protocol (see previous point 6). This part will be modified in the amended version of

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the ms.

7-> Were all specimens examined at the same magnification? Given that fluorescence brightness is exponentially proportional to magnification, it is important to use identical magnification in all cases.

Yes, magnification was always the same for all the specimens analyzed under the epifluorescence microscope.

8-> The means used to optically identify living specimens are not all inclusive: pseudopods are not always deployed by living individuals and some foraminifera do not consume algae (thus color can not be used as an indicator of health).

(Please see also Answers to DeNooijer, point 1). It is impossible to simultaneously test the same organism with multiple techniques (e.g., RB, CTG, calcein or MTT and FISH). As such, to discern live/dead foraminifera, we have used the only method (optical test) which was compatible with the further FISH testing of the same cell. In addition, several papers have shown that the “optical” approach for distinguishing live/dead organisms is widely utilised: Filipsson et al., 2010 (Biogeoscience, 7, 1335-1347); de Nooijer et al., 2008 (Limnol. Oceanogr. Methods, 6, 610-618 – “All specimens were screened for pseudopodial activity or presence of colored cytoplasm, indicating that individuals were alive at the start of incubation with HPTS”).

9-> In at least some environmental settings, rRNA can be preserved for considerable periods after an organism’s death (e.g., Coolen and Overman 2007 Environmental Microbiology; Panieri et al. 2010 Geobiology). The authors need to address this potential complication.

We are sorry but the Referee’s comment here is completely wrong. When citing the papers by Coolen and Panieri, the Referee confounds the 16S rRNA gene (which is, indeed, DNA) with the 16S rRNA (which is RNA, the transcription product of DNA). We know very well the papers by Marco Coolen whose title states: “217 000-year-old DNA sequences of green sulfur bacteria in Mediterranean sapropels and their implications for the reconstruction of the paleoenvironment”: the authors did not extract RNA from sediments, but the 16S rRNA gene (which is also called as “16S rDNA”). The same in Panieri. A more detailed rationale behind FISH, which conversely target the rRNA is presented in the letter to the editor as introduction to de Nooijer’s comment answers. In addition: while extracellular DNA is known to persist and to be preserved for considerable time in marine sediments (Danovaro and Dell’Anno 2005 Science), extracellular

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RNA is not believed to be so stable and is supposed to be degraded quickly.

10-> How useful will this method be for foraminiferal workers in general, given that some-times samples can not possibly be analyzed within one week (line 18 page 7481)? According to the FISH protocol, it is sufficient to perform the picking and the fixation step (addition of a mixture of PBS and ethanol, see paragraph 2.4 "Development of the FISH protocols", for details) immediately after sampling. After that, the appropriately stored samples can be immediately analyzed or, if desired, stored at -20°C .

11-> How can this method be used for deep-sea species that undergo huge pressure and temperature changes upon collection? Surely these foraminifera will die upon collection, and thus would give negative results when it is certain that they were living in the deep sea. This is an interesting point and of course it may happen. However, previous studies have demonstrated that benthic foraminifera originating from depths down to 2000 can be maintained in the laboratory at 1 atm (Hemleben and Kitazato 1995; Deep Sea Res I 42:827-832; Heinz et al. 2001; J For Res 31:210-227; Kitazato 1994 Mar Micropal 24:29-41; Kitazato et al. 1995; in: Biogeochem. Proces and Ocean Flus in the Western Pacific, H. Sakai and Y. Nozaki, eds. (Terra Scientific Publishing Company, Tokyo), pp. 331-342).

Moreover, Bernhard et al. 2006 (Paleoceanography 21, PA4210, doi:10.1029/2006PA001290) have tested foraminiferal metabolism of deep sea benthic foraminifera with the CellTracker Green (the principle of this method is the need for an active intracellular metabolism, which will produce the fluorogenic signal by enzymatically convert the non-fluorescent molecule). This physiological status could be even more sensitive to pressure changes than the ribosomes content. After that, it has been tested also the uptake of CellTracker Green directly in situ (Bernhard et al. Impact of intentionally injected carbon dioxide hydrate on deep-sea benthic foraminiferal survival, Global Change Biology (2009) 15:2078–2088). In our paper, we have only applied FISH to shallow water environments and the protocol has been proven to be unaffected by sampling at shallow depths. Future studies and experiments are needed to address the potential application of this protocol also to deep-sea forams.

12-> The Discussion grossly overstates the reliability and realities of the method (it is not cheap [reagents are expensive, the required microscope is expensive], it is not easy [there are many potential pitfalls for FISH on any taxon], and not quick [it still takes time to pick the specimens and then do an overnight preparation with many hybridization steps]!). Detailed comments on

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this problem appear among other issues listed below. In the amended manuscript, we will discuss all these aspects in more detail.

13-> Page 7475 Title: The title is misleading because the specimens subject to some of these protocols have been killed, thus can not be metabolically active. The work was done while Borelli was in Italy; she recently moved to USA. Thus the USA address should be noted as “current address”, not her primary address.

Thanks for this suggestion. We can change the title as suggested.

14-> Page 7477 The opening statement (lines 2-4) is not a sentence. The amended version will be carefully checked for this and other parts of the ms.

15-> Line 4: please define “hard shelled” Thanks for this request. Foraminifera with calcareous test and multilocular agglutinated. We do not consider among hard shelled, monothalamous transparent agglutinated species with a very thin walls (i.e. saccamminids and psammosphaerids) (Gooday, 2002, JFR 32, 384-399).

16-> Line 15: Murray and Bowser (2000) did not address the fate of “animal” tissue (Foraminifera are not animals). Further, Murray and Bowser is a theory paper, not a paper that actually demonstrated these results.

We will delete this reference.

17-> Page 7478 Line 3 states that CTG is time consuming, implying that the FISH method is not. This is not true: FISH takes just as much time, if not more, than CTG.

Right, please see above comments .

18-> Line 5: omit “s” on “controls”. Ok.

19-> Line 9: FISH is not “novel”; it has been in existence for at least a decade. Right. Our intention was to indicate that it has never been applied to foraminifera. We will modify the sentence.

20-> Line 15: add “of” between “study” and “prokaryotes”. “Prokaryote” is an antiquated term (see Pace, 2006 Nature).

We will replace the “term” prokaryote with “Bacteria and Archaea”. However, we do not fully

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agree with the Referee. Norm Pace's critique on the "prokaryote" term is a controversial consideration about the origin of the prokaryotic terminology. With the "prokaryote" term we refer to "those cells without a nucleus", which are either Bacteria and Archaea, but the term has not a taxonomical significance. In this sense, we believe it is of certain importance that the term "prokaryote" is still currently used on several top scientific journals (Nature, Science and PNAS) and on the most important microbiological textbooks.

21-> The stated goal (lines 28-29) that FISH is "more efficient, simple and rapid... than other available methods" is not addressed truthfully in the Discussion. Further, this assertion is highly debatable. The authors need to discuss the advantages and disadvantages of the method in the Discussion section (see above and below). FISH is not simple, not rapid, and not more efficient. Ok, please see above.

22-> Page 7479: What was the water depth where the samples were obtained? Line 5-6: How do the authors know the top 2 cm contained the highest abundance of foraminifera? There are many foraminiferal ecological studies that are based on the analysis of the first centimeters of sediment. In Bernhard et al., 2008 (Deep-Sea Research II 55: 2617–2626) studies, on the foraminiferal association of the Gulf of Mexico, both in bathyal and abyssal sediments, were conducted considering the 0-1 cm and 0-3 cm intervals. The remaining 75% of the core was fixed in formalin and archived. In Gooday, 1996 (Deep Sea Research, 10, pages 1345-1373) is reported that: "between 52.3 and 71.0% of the foraminifera within the top cm of sediment live in the upper 1 cm, 73.6-84.6% in the upper 2 cm". Reporting data from de Stigter et al., 1998 (JFR, 28: 40-65) who studied the bathymetric distribution of live benthic foraminifera along a shelf to deep sea transect in the Adriatic Sea, they suggest: "in all cores, the majority of stained foraminifera is found in the upper few cm of the sediment.."

23->What was the temperature at the field sites? How much did temperature change during the 2-4 hours of transport to the laboratory? What was the salinity, pH and carbonate chemistry of the sites? Line 19: How did the populations obtain oxygen? How might decreased oxygen availability affect results?

Data on the water temperature at the field site will be included. Temperature did not change significantly manner during the time transport to the laboratory. Following sediment collection, individuals were immediately put in airtight boxes previously filled with seawater from the same sampling site. Collected sediment did not show anoxic conditions, so individuals could have

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benefited of the oxygen dissolved in the water column, at the water-sediment interface, or in the pore water.

24-> Line 15: In microbiological terms, “culture” indicates reproduction (or division). This is not the case in this contribution and thus this term should be stricken from this contribution (also line 1 page 7480, etc).

We will change “foraminiferal cultures” in “foraminiferal maintenance under controlled conditions”. We want to specify that, at the moment of FISH tests, any reproduction event occurred in our laboratory cultural set up. Nonetheless after few months reproduction and growing of new individuals took place, in those that we could call as “proto-cultures”. These “new” individuals were utilized for experiments regarding anthropogenic effects on foraminiferal biology (Nardelli et al., in prep).

25-> Page 7480 Line 6: The authors likely intend to use the term “assemblage” rather than “association”.

Yes, the term assemblage will be used in the entire the ms.

26-> Line 17: Given the algal food offered to the foraminifera (*Dunaliella* and *Chlorella*, which are green), it is strange that yellow and light brown were used as distinguishing color characteristics. The specimens should have been bright green (see, for example, Barras et al., 2009 *J Foraminiferal Res*).

In de Nooijer et al., 2009 (*Bioscience*) it is reported that: “Before isolation, a small amount 10 of sediment was sieved over a 250 μm -mesh and the remaining material was screened for individuals of *Ammonia tepida* containing bright yellow protoplasm”. Moreover, direct observations show that, also in case of ingestion of green algae, after few hours, organisms return having a yellow or light brown cytoplasm colour.

27-> Line 21: Why were *Ammonia* and *Miliolidae* selected? We selected *Ammonia* and *Miliolidae* because they are the most representative of the shallow water environment studied in the Central Adriatic Sea. Moreover, on these two groups, there are some detailed and innovative studies, that contribute to a deeper biological knowledge of their cell metabolism and physiology (Bernhard et al., 2004, *Journal of Foraminiferal Research*, v. 34, no. 2, p. 96–101 – Use of the fluorescent calcite marker calcein to label foraminiferal tests -; Glas et al., 2010, *Forams*

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2010 Abstract Volume, pag. 94; de Nooijer et al., 2009, PNAS; de Nooijer et al., 2008, Limnol. Oceanogr.: Methods 6, 2008, 610–618 - Intracellular pH distribution in foraminifera determined by the fluorescent probe HPTS -; de Nooijer et al., 2007, Biogeosciences 4, 4 (2007) 493-504 - Copper incorporation in foraminiferal calcite: results from culturing experiments -; de Nooijer et al., 2006, The Journal of Foraminiferal Research; July 2006; v. 36; no. 3; p. 195-200 - Novel application of MTT reduction : a viability assay for temperate shallow water benthic foraminifera -).

28-> Page 7481 Line 9-10:Photosynthetic pigments always autofluoresce at proper excitation / emission wavelengths. If this was not observed then the authors were doing something wrong microscopically and/or the specimens had not ingested algae. Perhaps the authors intend to note that pigment autofluorescence does not exist at the wavelengths they used. The referee is right, we apologize for the lack of clarity. The organisms did not autofluorescence under the filter set utilized for FISH (which is the filter set appropriate for the fluorochrome utilized Cy3). This will be clarified in the amended version.

29-> Line 20: As noted above, specimens were air dried, so why does the fluorescent signal appear to include the entire volume of the shell (see Figures 4-5)? The foraminiferal cytoplasm, when dried, will not fill all chambers of the shells (cytoplasm is mostly water, and thus when dried, it will only fill a small fraction of the shell). The methods section (page 7481-7482) is far too detailed. It reads like a term paper or thesis. Please see comments above. The methods section will be shortened as suggested. The many details were intentionally provided as the method was new in this field. If the Editor will suggest to reduce this part we will be happy to follow the instructions.

30-> Page 7482 Line 12: Omit second “s” from “Specimens” Line 17: To test false positives of recently dead specimens, why were specimens dried for an hour when air drying was a step used in the actual FISH protocol (page 7481 line20)?

Please see comments above.

31-> Line 20-21: It is unclear why SEM was used given fluorescence is not detected with SEM.

SEM photos, because of their high magnification, have been used to help us in the interpretation of FISH results on shells. With the “Specimens washing” test, we wanted to test the possibility that the probe could hybridize also with the inner organic layer of the shell, the ec-

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toplasm or other organic residues of recently-dead organisms. Non-specific staining of FISH probe can sometimes occur under non-stringent laboratory condition, thus potentially providing false-positive results for dead organisms (Hugenholtz, Tyson Blackall, in "Methods in Molecular Biology"). This test, along with the results from the application of the "nonsense probes", allowed us to demonstrate that non-specific incorporation of the probe was not occur in the foraminifera. The SEM allowed us to be sure that the complete organic lining(s) were dissolved within one hour using our protocols.

32-> Page 7483 Line 1: "e" should be "y" in "microscope". Line 4-7: Why weren't the same measurements done on live vs dead specimens? Why was this only done on fed versus starved populations? We conducted experiments on fed vs starved population to demonstrate that there is a difference in the fluorescence emission between cells possessing a different metabolic status. We hypothesized that nourished individuals were more active than starved ones and thus should have emitted a higher fluorescence signal, as a result of a higher number of active ribosomes in the cells (which are busy in synthesizing proteins for sustaining cell growth). This is to our opinion an important experiment allow to strongly demonstrate that the intensity of the fluorescence signal is dependent from the metabolic status of the foraminiferal cell. A similar experiment has previously been carried out on planktonic protists (see Lim et al 1993 Appl Environ Microb). Analogously, Rice et al. 1997 (Appl Environ Microb), who applied FISH to study marine protists, observed that cell fluorescence intensity increased in cultured specimens and concluded that fluorescence intensity allowed inferences about cell growth rate. In this sense, a comparison between live versus dead cells cannot be performed given that dead cells will provided (as stated above) null or low-background fluorescent signal.

33-> Page 7484 Line 4-7: Did doubling the time in NaClO make any difference? Why is this data not shown? It appears that the authors merely assumed it made a difference. We doubled the time of NaClO treatment to obtain a more efficient removal of organic residues from shells. In that way, wanted to test FISH protocol on shells, however this part is not essential and will be omitted on the amended version of the ms.

34-> Line 9:Please define where coastal sediments were obtained (are these equal to Falconara and Portonovo?) Yes, we will clarify this sentence.

35-> Line 10:Most people would call FISH a probe that hybridizes, it is not a "stain". Ok, we will change accordingly.

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36-> Line 11-15: This repeats what is stated in the Methods, it should be omitted. Ok, we will change accordingly.

37-> Line 13: Proper spelling is “species” (there is no such thing as a “specie”). Ok, we will correct accordingly.

38-> Page 7484, Line 15-22: As noted above, this section is uninterpretable. The section will be revised to make it more clear to the readers.

39-> Table 3 is also not clear. Of these 19 Ammonia, how many were living? Although the text states that 17 emitted fluorescence, the text does not say if they were live or dead as determined by optical means. The same question arises for the other 7 that were incubated with S1 7. Thus, it is impossible to know the efficacy of this method. The Table will be changed to make clear all of these aspects (please also see above).

40-> Page 7485 Line 3: Perhaps the authors intend to state “. . . agglutinated particles in the shell...”? Ok, we will correct accordingly. Line 9-15: As noted, it is extremely peculiar that the fluorescence was homogeneous in the “protoplasm” given these specimens were dried. Imagine a grape vs a raisin. It is impossible that dried cytoplasm would have remained homogeneously distributed in dried specimens. Thus, the fluorescence cannot represent foraminiferal cytoplasm. Please see above and the letter to the editor as introduction to de Nooijer’s comment answers.

41-> Line 22-23 requires a literature citation. An appropriate literature citation will be included.

42-> Line 27: It is unfathomable that this part of the study relied on 3 specimens. Perhaps the authors intend to state that pooled samples were analyzed? If so, how many specimens per sample? This point requires distinct clarification. Additional analyses will be carried out to implement the dataset.

43-> Page 7486 Line 13 and 15 should state the number of individuals rather than percentages. In reality, only 5-6 specimens were living when using color and pseudopodial extension as criteria and only 4-5 fluoresced. These are such small numbers that this entire paper is flawed. As above, additional analyses will be carried out.

44-> Line 22: Bernhard (2000) discusses terminal versus non-terminal methods. The cited reference will be included.

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45-> Page 7487 Somewhere near line 2 the authors need to state that their method is a terminal method, and thus their arguments should have no impact on non-terminal method assessments. Also, it must be noted that one of the major advantages of CellTracker Green method is that it can be used as a non-terminal method. The sentence will be modified accordingly.

46-> Line 7: The sentence should end in "... as a live-dead indicator." Ok, we will correct accordingly.

47-> Line 8: "were" should read "was". Ok, we will correct accordingly.

48-> Line 14-17: The qualifier "non-fixed" needs to be included in this statement because it is established that chemical fixation with formalin or other aldehydes causes some autofluorescence at Cy3 wavelengths. (Please see above point 28). We have not used formalin neither other aldehydes as fixative, but ethanol and PBS. We will perform additional autofluorescence tests to verify our data.

49-> Line 19: "(i.e., the intracellular. . ." must be changed to "(i.e., our intracellular. . ." because TEM distinguishes unequivocally live from dead and this intracellular method should not be considered equivalent to the author's "intracellular analysis" by merely assessing color. Ok, we will change accordingly.

50-> Line 1 7-19: The meaning of this statement is unclear. The authors thus need to clarify it. It is impossible to comment on its content. Line 28: This sentence needs to cite Bernhard et al. (2006) which showed this. Thus, the statement should read ". . .marine sediments, as noted by Bernhard et al. (2006)." Line 29: "the use of the term "reliable" is unacceptable. This method has not been shown to be reliable, given the extremely small population sizes and severely limited species composition. We will clarify the sentence.

51-> Page 7488 Line 7: "die, his cellular" should read "dies, its cellular" (foraminifera do not have gender). Ok.

52-> Line 9-23: The assertion that this method is "reliable" is unfounded, the assertion that the method is "much less time consuming" is also unfounded since FISH takes quite a bit of time. Specimens still need to be picked and the lab work is intensive. Plus, an epifluorescence microscope, which is not inexpensive, is also required for this method. We will clarify that the FISH, analogously to the Cell Tracker Green method, needs an epifluorescence microscope to

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be performed (which is not un-expensive).

53-> The authors need to discuss exactly how much time and cost is involved with this method and how long it took them to actually get it to work. Not all species should be expected to respond the same way, so each laboratory will have to repeat the same pitfalls. This paper presents the method as “easy”, which is an overstatement. And, the method certainly should not be reported to work for all foraminifera, given only about 4 species have been tested. There are many species with opaque shells.

More information will be included in this sense. Of course are data are not based on a large dataset, but we will include more analyses on the amended manuscript, which will possibly include representatives of other foraminiferal groups.

54-> What about for deep sea species that undergo huge pressure and temperature changes upon collection? Surely they will die upon collection, and thus would give negative results when it is certain that they were living in the deep sea. Please see above, point 11.

55-> Page 7489: This entire page needs to be rewritten in light of comments throughout this review. Ok.

56-> The manuscript would benefit greatly from the expertise of a native-English speaking individual. See, for example, page 7483 lines 13-25m which are particularly awkward and undecipherable.

Ok, this will be done. When stating “both cultured and wild dead foraminifera. . .” (page 7483, line 13) we were meaning that we tested the FISH protocol on dead organisms both from cultures and from natural sediment samples. However we will use more appropriate terms

57-> Table 1: What does “NO” mean in the column headed “Autofluorescence assay”? Does NO = nitrous oxide? Does NO= no (if so, what does that mean exactly? The assay was not done? Why have this column if all species were treated the same way?) . The table will be revised and better presented as requested (see point 3).

58-> Table 3: Define the difference between zero and “none”? See previous point.

59-> Figure 2: What does “particular” mean in the caption for B and F? Figure 3 caption should read “Little fluorescence occurs” instead of “No fluorescence occurs” given that the specimen can be seen with epifluorescence.

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The term “Particular” should have been “zooming”. However, the entire ms will be checked by a native English speaking. For background fluorescence, please see above (response to point 1).

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