Joint Department of Biomedical Engineering
The University of North Carolina at Chapel Hill and
North Carolina State University at Raleigh

152 MacNider Hall, Chapel Hill, NC 27599-7575
(919) 966-1175; (919) 966-2963 fax
http://www.bme.unc.edu

2147 Burlington Laboratories, Raleigh, NC 27695-7115
(919) 515-5252; (919) 513-3814 fax
http://www.bme.ncsu.edu

December 2, 2010

PLoS Biology
Public Library of Science
185 Berry Street, Suite 3100
San Francisco, CA 94107

Dear PLoS Biology editors:

Please consider this revised manuscript titled “High-Resolution Quantification of Focal Adhesion Spatiotemporal Dynamics in Living Cells” by Matthew E. Berginski, Eric A. Vitriol, Klaus M. Hahn and Shawn M. Gomez for publication in *PLoS Biology.* We note that this is a previous interaction (10-PLBI-RA-6319). We greatly appreciate the reviewer’s thoughtful comments and have tried to thoroughly address their questions and concerns.

Highlights of some of our changes relative to specific reviewer comments follow below. A full, point-by-point response is included after this summary section:

- Reviewer 1 had a major concern regarding the availability of the software, stating that “*Taken together, I would recommend acceptance of the paper, under the condition that the software is made available to the scientific community (ideally – downloadable from the Journal’s website).*”
    - We were not clear enough in our original submission and have clarified it in this revision – the software is freely available under an open-source license and can be downloaded from our lab website (http://gomezlab.bme.unc.edu/tools) and includes documentation and sample data on which the software can be run. In addition, we would be happy to have the software available on the PLoS’ website. Continued updates to the software will be made available on the lab website.
    - A number of other concerns were also addressed.
- Reviewer 2 was perhaps most interested in us elucidating the mechanisms and role of the phosphorylation event on Paxillin that we describe. While we do agree that this is a very interesting and important area worth pursuing, it was not the main focus of this manuscript. We have addressed all other Reviewer concerns as well as possible and have further altered the title to deemphasize the “key regulator” aspect of the S178 Paxillin mutant used in this work.
- Reviewer 3 was very favorable towards the work, with the primary critique being to provide supporting measurements using another adhesion marker. We used FAK as an alternative marker of adhesions, replicating relevant aspects of the statics and dynamics analysis using this marker. We found identical rates of assembly and disassembly for adhesion regardless of whether Paxillin or FAK was used as the adhesion marker.

Again, we greatly appreciate the reviewers' comments and questions and feel that they have greatly improved the quality of the manuscript. We believe this work provides a novel picture of adhesion dynamics in living cells as well as a tool for use by the broader adhesion research community. We look forward to moving ahead towards publication of this manuscript.

Sincerely,

Shawn M. Gomez & Klaus M. Hahn
School of Medicine, UNC-Chapel Hill

Detailed Response to Reviewer Comments

Reviewer #1:
Remarks for the Author:
In this paper, the authors present an elaborate study involving focal adhesion (FA) segmentation and quantification, as well as tracking of FA dynamics in live cells. They apply their analysis software for the characterization of the effects of mutating a major phosphorylation site in the FA protein paxillin on the structure, distribution and fate of the adhesion sites. The work presented here is, certainly, of high technical quality, and could potentially be useful for researchers working on FA dynamics. On the other hand - the novelty of this paper is rather limited, since FAs were segmented before, using a similar basic algorithms ("watershed-based"), and used for quantification of FA features in both fixed and live cells. The authors introduce a number of significant additions in the experimental design (e.g. use of TIRF illumination), as well as in the software, but whether these improvements are significant enough to justify a full publication - is questionable.

- We appreciate the positive comments, and do feel that there are multiple areas of novelty. From the computational standpoint, it is true that methods have been developed for identifying and quantifying FA features in cells. However, integration of identification, segmentation, tracking and analysis has not been done before for adhesions in live cells and this provides important new information and a new way to analyze adhesion function. Through this integration we have been able to quantify the dynamics of individual adhesions, as well as adhesion populations, on a scale that has not been previously attainable. Finally, we feel that this approach allows for the characterization of novel biology. For instance, in Figure 4 we are able to reveal relationships of assembly/disassembly in a spatial context, something that has not been demonstrated before. Similarly, demonstrating shifts in the position of an adhesion's place of birth due to a point mutation as well as other properties that we have quantified is inherently novel. Admittedly, further study is needed to provide a greater level of biological understanding. We hope that what we have shown some of this novelty as well as significant future potential.

The choice of paxillin mutation in S178 is a good one for demonstration of the power of the imaging the software - but the data shown here do not provide a meaningful contribution to the understanding of the role of this phosphorylation in regulating FA properties and dynamics, and merely serve as good demonstration of the quality of the software.

- We certainly agree that as a biological study, the work is by no means complete. However, we feel that our study does make a contribution to understanding the mechanism through which phosphorylation of Paxillin at Serine 178 affects adhesion behavior. One of the major goals of this work was to develop a system that could detect and quantify changes in adhesion properties that are difficult or impossible to do with current approaches. Properties having subtle changes, involving only specific subpopulations, or changes in dynamics and/or spatial distributions often fall into this category. A primary goal with the Serine 178 mutation was to make a small perturbation to a single adhesion constituent to see how adhesion parameters were affected globally. A further rationale for this mutation choice is that this mutation was previously (and only qualitatively) described as having a strong effect on cell motility. To our knowledge, this is the first work that helps to explain why this change in motility may occur: through significant decreases in adhesion rates of assembly and disassembly. The finding of a spatial shift in adhesion birth position due to this mutation is also very intriguing, but awaits further specific investigation

Beyond these issues, the authors need to address a key problem associated with the segmentation of such structures as FAs - namely the basic definition of FAs. It is not clear what are the features on a paxillin-containing structure (intensity, size, shape) that qualifies it as a "focal complex" or "FA".

- The defining of what qualifies as a focal adhesion is a fundamentally tricky one as it is dependent on aspects such as the cell type, experimental setup as well as differences in definition between research groups. For instance, the Geiger group recently used a related imaging approach and siRNA screen to investigate FA properties within HeLa cells and defined an adhesion as “all distinct paxillin-containing structures larger than 0.25 sq microns (Winograd-Katz et al., 2009). In this manuscript, we avoid making the distinction between focal complexes and focal adhesions as the exact parameters that differentiate the two are based not only on size, but also on molecular composition. The Geiger group has even gone so far as to subcatagorize Focal Complexes into two groups (“Early” and “Late” Focal Complexes) based on their constituent components (reviewed in Zaidel-Bar et al. 2004). We simply state that we are able to examine the dynamic of adhesion complexes. These will of course be within a certain size range, determined by the limits of the microscopy technique used. Our goal in this study was to develop methods to obtain a completely unbiased survey of the adhesion profile of a migrating cell. We can identify any subpopulation of paxillin-containing structures based on size, shape, intensity, position in the cell, and dynamics (in any combination of the above variables), but in order to call these structures focal complexes, we would have to verify the presence (or absence) of certain adhesion components. This could be done with multi-color/ratio imaging and is a direction we plan on taking future research, but

would be a study unto itself and outside the scope of this manuscript. We did perform an extensive sensitivity analysis and found that derived properties (e.g. the rates of assembly and disassembly) are not dependent on specific thresholds used to define and segment individual adhesions.

In conclusion - the technology looks good and potentially very useful for researchers (provided that the software is available for them), yet the novelty is limited. Taken together, I would recommend acceptance of the paper, under the condition that the software is made available to the scientific community (ideally - downloadable from the Journal's website).

- We realized that we had not made software availability explicitly clear in our earlier manuscript. The software is provided under an open source license and can be downloaded on our own website (http://gomezlab.bme.unc.edu/tools) where improved versions will also be provided. We would be happy to have it downloadable from the Journal's website as well.

Reviewer #2:
Remarks for the Author:
This manuscript describes a software application for the "unbiased" analysis of the position and dynamics of "all" the focal adhesions within cultured cells. This approach is reported to shorten data analysis times and avoid user-dependent selectivity issues. The focal adhesion protein paxillin and a previously described phospho-mutant were used in the analysis to provide proof of principle. Evidence is also presented that mutation of the S178 residue results in diminished focal adhesion turnover and position within the cell that could account for previously reported defects in cell migration. This is useful technology but there is little in the way of new mechanistic insight.

- The approach is as unbiased and comprehensive as we can readily imagine making it – labeled adhesions must have an intensity above a given level above background for them to be recognized, they are all tracked and their characteristics quantified in an equivalent manner.
- We feel that this method does far more than just shorten analysis times and avoid user-dependent selectivity issues. While in theory one could analyze the over 160,000 adhesion lineages measured across more than 5000 images, we feel that this not practically feasible. In addition, by comprehensively quantifying adhesions in live cells, we are able to establish adhesion properties that have not been defined previously, such as spatial position at birth and death. Furthermore, the ability to accurately measure large numbers of adhesions allows potentially subtle phenotypic changes to be analyzed in a statistically rigorous manner. The described approach should greatly enhance future focal adhesion studies.
- We do realize that there is much that needs to be done to use the new methodology to obtain mechanistic understanding of focal adhesion behavior and dynamics. We feel that the described approach provides a new tool that could aid many researchers in these efforts. This manuscript describes this tool (freely available), shows its performance on

real data sets and its validation through simulated data sets, and provides an analysis of the S178A paxillin mutant showing the strong effects of this mutation on adhesion turnover with little change in other properties. It also indicates intriguing changes in adhesion dynamics (e.g. the shift in birth position due to the inability of JNK to phosphorylate S178) that warrant further studies. We plan to use this tool to understand underlying mechanisms in future experimental efforts.

Specific points
Cells expressing ectopic protein are used in this study, requiring multiple rounds of infection. Blots showing expression levels of WT and S178A as they relate to endogenous protein need to be presented. Small changes in adhesions that re observed could be due to expression differences.

We shared this concern as well – that the differences found between the Paxillin-EGFP and Paxillin$_{S178A}$ were potentially caused by a differential expression of the two fluorescently labeled adhesion components. To address this issue, we determined whole cell, cell background, and average adhesion fluorescence and determined all three parameters have no statistically detectable difference for cells expressing Paxillin-EGFP and Paxillin$_{S178A}$-EGFP. Thus, differences observed between the two cell lines are not due to differential expression. We have added Figure S3 to the publication showing these results.

Representative images of both sets of cells should be provided in order to valuate focal adhesion distribution. Movies used in the data acquisition should also be provided so we can evaluate the phenotype and dynamics of the cell over the extended time frame. How much photobleaching occurs during the course of a 4 hour TIRF movie? Is this correction incorporated into the intensity analysis?

- We have provided representative images as well as movies. The degree of photobleaching was assessed and was found to not be an issue. Regardless, correction for photobleaching is part of the standard image analysis process used in the manuscript.

It is hypothesized that the adhesions arise at the actin lammellipodia-lammella interface and that this position is changed by the S178A mutant. The authors are well qualified and equipped to test this with a fluorescently labeled actin probe and this should be done.

- The reviewer recommends conducting an experiment that would further support our observations concerning the underlying actin network changes around the shift from the birth to death adhesion regions. While we do feel this experiment would be highly interesting, the detailed study of the spatial distributions of adhesions under different conditions would require a separate dedicated study. We have tried to focus in this work solely on the characterization and quantification of adhesion dynamics. In future work, we plan to investigate the relationship between the spatial aspects of adhesion dynamics

and key structures (e.g. at the lammellipodia-lamella interface) in normal adhesion behavior.

The mutant cells require 3 hrs to spread before imaging, but the WT cells are imaged after only 30 min. Are these really comparable? The WT cells are probably still spreading at this time. Thus any differences in distribution/turnover that are calculated may be more representative of the spreading status of the cell rather than the effect of the mutation. Cell populations should be spread for an extended period of time (overnight?) to reach equilibrium before they are imaged.

- We wanted to image the cells during migration, when adhesion turnover is highest. In our hands, these MEFs exhibit the best migratory phenotype 0.5-8hrs after being plated on fibronectin (right after spreading). It should be noted that while the S178A cells were given an extra 2.5 hrs to spread, the window in which they were imaged, 3-8hrs after plating, overlaps completely with the window in which WT cells were imaged (0.5-8hrs). Furthermore, in any given population of cells, there is a heterogeneous distribution of cells in different migratory states (spreading, migrating, quiescent, etc.). All cells were chosen for imaging based on their phenotype- a cell that had completed spreading and was now undergoing migration. Allowing the cells to sit overnight would result in cells with reduced migration, more quiescence, and less adhesion turnover.

I am not convinced that "all" adhesions are being imaged without user bias. Indeed, the authors clearly state they ignored short-lived adhesions-why? Are these not important to cell function? Also, what is the minimum size of adhesion can they detect/resolve with 2X2 binning? Furthermore, on P.18 it is reported that the user inserts a "specified length" in the calculation of assembly/disassembly.

- We filtered out short-lived adhesions only for the assembly and disassembly studies. The reason for this is that accurate quantification of assembly and disassembly requires that we see the process from beginning to end – from the birth of the adhesion all the way to it's death. Furthermore, to get accurate measures of assembly and disassembly, we need to have a sufficient number of measurements, both on the rise and fall of these curves, to make accurate fits to the data. As a result, we chose to require a minimum of 10 consecutive measurements of adhesion assembly, 10 consecutive measurements for disassembly and any number allowed in between. The "specified length" refers to this minimum of 10 observations in the assembly/disassembly phases. We also included a requirement to have observations of the adhesions' birth and death. Thus adhesions that could satisfy these criteria had to have a minimum lifetime of 20 minutes and short-lived adhesions were those that fell below this value. All other measures do not have this requirement and thus all adhesions are measured in these cases (given that they are of sufficient intensity relative to background to be detected and tracked). We do think that short-lived adhesions are important, but the accuracy of rate measurements requires us to place some conditions on the amount of data needed for fitting.
- The size of each pixel is 0.215 microns in length and since we report and track single pixel adhesions (after image filtering), the minimum size adhesion we can detect is 0.0462 square microns.

More mechanistic data is needed regarding how the S178A mutation affects adhesion dynamics. Otherwise these observations represent an incremental advance at best regarding our understanding of how phosphorylation of this residue affects paxillin function. The title of "key regulator" is overstated in the regard.

- We were not specifically clear enough on this point and our title was not correct in this regard. We have changed the title and demphasized the “key regulator” issue mentioned. Our goal in this work was not to focus on this particular mutation or explain how phosphorylation of this residue affects paxillin function. Instead, we have focused on describing and validating a system for the analysis of focal adhesion spatiotemporal dynamics in living cells. As discussed in our response to Reveiwer 1, the use of the S178A mutation is a highly relevant system perturbation, with clear effects on global cell motility. We do realize that we have in no way explained all the mechanistic details of this mutation and its role in motility. We do, however, feel that it conclusively shows and quantifies previously undescribed effects of this mutation on adhesion dynamics. These results do help to explain previously described observations. Much work undoubtedly remains in describing the role of this phosphorylation site in adhesion dynamics as well as in other signaling events. We hope that this computational platform will help investigators studying this and related systems.

Detailed information regarding the statistical analysis is required. Some data sets seem to have extraordinary errors yet are show as being highly significant. For, example, in fig 5 mean area is less than 0.5 um but the errors are up to 14 in both the WT and S178A populations.

- We have added a “Statistical Tests” section within Methods and have clarified Figure 5, making it identical with all other box and whisker plots. For Figure 5, the top line of the box gives the 3rd quartile range and the whisker extends up to 1.5 times the interquartile range. Thus these are not really “error bars” but rather give a picture of the distribution of the data. An example of interpreting these would be that three quarters of the WT adhesions (panel A) have area less than 0.8 square microns (everything below the top line of the box). The bold central line gives the median value. Each of these bars is composed of over 300,000 data points and thus it is possible to detect subtle differences between distributions. We used a non-parametric Wilcoxon rank-sum test to assess the differences between distributions.

For this to be useful to the scientific community at large the authors need to provide more details regarding the software used and its availability.

- As discussed in response to Reviewer #1 above, we are making the complete software package available under an open-source license.

Reviewer #3:

Remarks for the Author:

This manuscript describes a new analysis system for the detection, tracking, and data extraction of adhesions in living cells. The analysis system is applied to quantifying the dynamics of the adhesion protein paxillin using total internal reflection fluorescence microscopy (TIR-FM). The authors analyze the size, shape, and intensity of paxillin- containing adhesions over time. They show that a paxillin mutant (S178A) affects the size, distribution, and rate of adhesion assembly.

This is a very nice study that develops a new method for quantifying the spatiotemporal dynamics of adhesions. The described method automates the collection and quantification of adhesion dynamics, making it feasible to analyze large numbers of adhesions, and should be of great interest and benefit to the research community.

- Again, we appreciate the positive comments and believe that this platform (freely downloadable and under an open-source license) will be of broad utility to the research community.

The data presented are convincing and show the feasibility and validity of the approach. However, the manuscript in its current form focuses more on technology development than on biological application. In addition, since on one adhesion protein (paxillin) was analyzed, their data indicates properties of paxillin assembly and disassembly, but cannot necessarily be extrapolated to adhesion assembly/disassembly in general. To address both of these concerns, the authors should analyze the spatial properties of the assembly and disassembly of another adhesion protein, such as vinculin. This analysis will strengthen this manuscript and allow the authors to provide a more general picture of the spatiotemporal dynamics of adhesions, which will increase the impact of this study.

Comments:

1. *In Figure 3A, is the scale bar 10 □m or 10 mm as indicated in the figure legend?*
   - It should be 10 microns – this has been corrected in the text.

2. An analysis similar to that done in Figure 4 should be performed with another adhesion protein, such as vinculin.

We have addressed the reviewer's concerns by adding Focal Adhesion Kinase (FAK), another key adhesion molecule, to our analysis. We did not have myr-RFP for our EGFP-FAK cells, which meant that edge detection had to be done through computational means. While we did do this, the results were noisier, so differences could not be confidently assessed for significance. In the end, we decided not to include the spatial comparison with FAK in this manuscript. Future work will address more fully the spatial dynamics of adhesions and adhesion components under different conditions/perturbations. Regardless, all other analyses could be performed and we found identical rates of assembly and disassembly for both Paxillin and FAK, and slight differences in their static properties (Figures in manuscript and supplemental materials). These results further support the methodologies and results found with the initial Paxillin-based experiments.