Reducing background fluorescence reveals adhesions in 3D matrices

Kristopher E. Kubow¹ and Alan Rick Horwitz¹,²

To the editor:

Adhesion complexes in cells growing on planar substrates have been studied for over three decades. From these studies, several classes of adhesions have been described based on size, location, morphology, dynamics or molecular composition, and their dual role as signalling centres and linkages that connect the extracellular matrix (ECM) with the cytoskeleton has also emerged¹. In contrast to this large and growing understanding of adhesion on two-dimensional (2D) substrates, little is known about the adhesions formed during three-dimensional (3D) growth of cells, including whether they even exist. Immunostaining and microscopy of fixed samples²–⁸ and dynamic imaging at low spatiotemporal resolution⁹ have shown the presence of large, elongated adhesions on cells in various 3D model systems. However, attempts to visualize adhesions in living cells growing in 3D with resolution similar to that routinely used in 2D have not been successful¹⁰. This has led to the recent conclusion that the adhesions defined and characterized in 2D cultures either do not exist in cells in 3D, or are too small or short-lived to be observed¹¹.

One major limitation of imaging live cells in 3D culture is background fluorescence caused by the overexpression of genetically encoded, fluorescently tagged proteins. Having saturated all available association sites, excess fusion proteins accumulate in the cytoplasm resulting in diffuse background fluorescence. In cells on 2D substrates, this cytoplasmic background is less detrimental because of the thinness of the lamellae in migrating cells. Moreover, background fluorescence can be reduced in 2D studies by using TIRF (total internal reflection fluorescence) microscopy, which excludes fluorescence above approximately 100 nm from the substrate. In contrast, protrusions formed on 3D matrices may be thicker¹² and must be visualized using wide-field, confocal or multi-photon microscopy, which section a minimum thickness of 500–800 nm (ref. 13). Therefore, overexpression of an adhesion-specific fluorescently tagged protein can be more detrimental to imaging in 3D than in 2D and result in diffuse cytoplasmic fluorescence that masks the signal of molecules localized to adhesions.

To address this issue, we used an EGFP (enhanced green fluorescent protein)–paxillin construct under the control of a truncated CMV (cytomegalovirus) promoter that was originally developed to express GFP-β-actin at very low levels¹⁴. The truncated CMV promoter reduces expression from the plasmid and therefore results in lower protein levels than those achievable by simply minimizing plasmid copy number. U2OS osteosarcoma cells were transfected with EGFP–paxillin in this vector, seeded within fibrillar collagen gels, and imaged with a laser scanning confocal microscope between 3–5 h after seeding. The collagen fibres were simultaneously imaged in reflectance mode. All images were taken several millimetres from the lateral edges of the gels. The cells were mostly multi-polar with three or more protrusions of variable size and shape that extended out from the cell body, spanning multiple focal planes, and exhibited numerous adhesions (Fig. 1a and Supplementary Information, Fig. S1a–f).

Adhesions were rarely observed in areas more proximal to the cell body; however, as these

---

¹Department of Cell Biology, University of Virginia, Charlottesville, VA 22908, USA. Correspondence should be addressed to A.R.H. (e-mail: horwitz@virginia.edu)

© 2011 Macmillan Publishers Limited. All rights reserved.
CORRESPONDENCE

Figure 2 Dynamics of cell–matrix adhesions in 3D culture. Frames are taken from Supplementary Information, Video S1. The top row shows Z-projections of a protrusion end from a U2OS cell that was transfected with a plasmid encoding promoter-truncated EGFP–paxillin. The bottom row shows the image from the top row (green) overlayed with a reflectance image of the collagen fibres (magenta). Time indicates the min:s since the beginning of the movie. At 0:20, a small adhesion (arrowhead) moves rearward, pulling a collagen fibre. At 2:30, a new protrusion (boundary shown by dotted line) pauses, two new adhesions form at the leading edge (arrows), and the earlier adhesion (arrowhead) has elongated while travelling rearward. At 3:10, the two new adhesions (arrows) continue to grow and translocate with the attached collagen fibres. By 4:50, the new adhesions have continued to grow and move rearward while the early adhesion (arrowhead in previous panels) is no longer visible. Scale bar, 2 μm.

areas of the cell were generally much thicker than the distal ends of the protrusion, and thus had higher cytoplasmic background fluorescence, we cannot exclude their existence. We observed adhesions in cells at depths up to the limit of the working distance of our objective (approximately 350 μm). Regardless of depth, at least 50% of the cells with a detectable level of the low-expressing EGFP–paxillin exhibited adhesions (Supplementary Information, Fig. S1g, m). Cells in which adhesions were not visible tended to have fluorescence intensities that were either relatively high (high background) or relatively low (low overall signal; Supplementary Information, Fig. S1h, n).

High-spatial-resolution imaging of the protrusions revealed distinct adhesions (Fig. 1b, lower panels) with a median length of 1 μm (0.8–1.45 μm, 25th–75th percentiles; n = 72 adhesions). Adhesions were also observed in similar short-term experiments with HT-1080 cells (Fig. 1b; median 1.2 μm; n = 36), with both cell types expressing EGFP–vinculin from the same vector (Fig. 1c), and in overnight cultures of U2OS cells (data not shown). In contrast, U2OS cells transfected with low levels of EGFP–paxillin expressed under the full-length CMV promoter, or even with high levels of the truncated promoter plasmid, had a diffuse cytoplasmic fluorescence similar to that of a fluorescent protein expressed alone (Fig. 1b, upper panels). Finally, we observed adhesions in HT-1080 cells cultured overnight in rat-tail collagen gels (Supplementary Information, Fig. S1i–n).

To image adhesion formation, growth, and disassembly, we acquired time-lapse Z-stacks of protrusions from U2OS cells cultured 3–5 h as in Figure 1. As highly dynamic nascent adhesions form and disassemble rapidly with an average lifetime of about 1 min, we imaged with a 10 s time-interval to be sure of capturing short-lived adhesions. Figure 2 shows frames from a representative time-lapse movie of the protrusion of a U2OS cell transfected with a plasmid encoding the promoter-truncated EGFP–paxillin (Supplementary Information, Video S1). The protrusion undergoes cycles of extension and adhesion formation followed by rearward adhesion movement and matrix fibre deformation. Similar observations were made in six independent experiments and with HT-1080 cells (Supplementary Information, Video S2).

In general, new adhesions formed along collagen fibres at the leading edge of protrusions and travelled rearward, causing fibre deformation. The adhesions had an initial diameter of 0.4 ± 0.07 μm (mean ± s.d.), placing them in the range of focal complexes, but larger than diffusion-limited nascent adhesions. Over the course of the movies, most translocating adhesions grew in intensity and length; adhesions that did not translocate were stable, apparently maintaining isometric force on the matrix. Treatment with a combination of the Rho kinase (ROCK) inhibitor Y-27632 and the myosin light chain kinase (MLCK) inhibitor ML-7 decreased adhesion size similar to their effect in 2D (ref. 1).

Our observations demonstrate that cell-matrix adhesions can form in 3D collagen matrices, show that their dynamics can be studied in living cells, and support previous observations by immunostaining and microscopy in collagen, fibrin, and cell-derived 3D ECMs. In addition to reducing background fluorescence, factors that affect adhesion size (and therefore signal intensity) may affect adhesion detectability. For example, microenvironment stiffness will affect cell contractility and thus adhesion size and composition. Therefore, more-pliable regions of collagen gels may have smaller and consequently less readily detected adhesions; if so, it would point to pliability, in addition to dimensionality, as the determining factor. In addition, adhesions are difficult to visualize in certain cell types. For example, leukocytes do not show prominent adhesions on ICAM-1 coated substrates and can migrate in an integrin-independent manner. The effects of cell contractility on adhesion phenotype have been well-studied in 2D systems; future work will need to determine how these interactions occur in 3D. Reducing background fluorescence overcomes an initial barrier to adhesion visualization in 3D and allows studies of the influence of 3D topography, pliability, matrix protein composition, and cell contractility on adhesion phenotype.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology/

Note: Supplementary Information is available on the Nature Cell Biology website
ACKNOWLEDGEMENTS
We thank C. Choi, M. Vicente-Manzanares, R. Tilghman, and E. Gratton for helpful discussions and technical assistance. The research was supported by the Cell Migration Consortium (U54 GM064346) and GM23244. K.E.K. is supported by a Cancer Training Grant at the University of Virginia Cancer Center (T32 CA009109-34).

AUTHOR CONTRIBUTIONS
K.E.K. performed the experiments and analysed the data. K.E.K. and A.R.H. designed the experiments and wrote the paper.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

METHODS

Fluorescent protein constructs. The EGFP–paxillin plasmid has been described previously. The EGFP–vinculin plasmid was a gift from S. Craig (Johns Hopkins, USA) and the CMV-promoter-truncated EGFP–β-actin plasmid was a gift from T. Mitchison (Harvard Medical School, USA). All constructs were in Clontech pEGFP vectors. The promoters of EGFP–actin and EGFP–paxillin were excised by digesting with Asel and Nhel. The truncated CMV promoter from EGFP–actin was then ligated into the digested EGFP–paxillin plasmid. The same procedure was used to make promoter-truncated EGFP–vinculin, except ApaLI was used instead of Asel due to the presence of a duplicate restriction site within the vinculin gene. The TagRFP-T plasmid was a gift from R. Tsien (UCSD, USA).

Cell culture and transfection. U2OS cells were cultured in McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS) and were transfected with Lipofectamine 2000 (Invitrogen) or TransIT-2020 (Mirus Bio). HT-1080 cells were cultured in minimum essential medium (MEM) with Earle’s Salts, non-essential amino acids and 10% FBS, and were transfected with Lipofectamine (Invitrogen) or TransIT-2020. During experiments, the cells were cultured in CCM1 + 10% FBS to attain a final volume of 300 μl of the field-of-view was selected so that one entire depth-slice was acquired every 0.33 μm (the axial resolution was determined empirically to be approximately 0.75 μm). To image the leading edge of a typical protrusion (and pad the top and bottom with additional slices to account for Z-drift over the course of the experiment) 10–20 Z-slices were necessary. The shortest possible pixel dwell time (2 μs per pixel) was used. A smaller sub-region of the field-of-view was selected so that one entire Z-stack could be completed within 10 s. Z-stacks were acquired every 10 s for a total of 5 minutes. Laser power was adjusted to minimize photobleaching. Cells selected for imaging were not near or at the glass surface (except when acquiring data for the data shown in Supplementary Information, Fig. S1g, h, m, n) and were not in close proximity to other cells.

For the data shown in Supplementary Information, Fig. S1g–n, we performed experiments in which all fluorescent cells in a given area were imaged and the depth of their lowest (closest to the glass) point was recorded. The imaging area was several millimetres away from the lateral edges of the gel and covered a depth ranging from the glass surface (0 μm) to the limit of our ×60 water-immersion objective (approximately 350 μm). Images were given generic names and the depth data were kept in a separate file as to exclude any visible adhesions) was measured. Intensity measurements were performed with the FV10-ASW Fluoview software (Olympus) and were corrected for additional background noise by subtracting the mean intensity of a region drawn outside of the cell. All measurements were performed blind with respect to the depth data (collected during acquisition; see above) and intensity measurements were performed after counting the adhesions in order to prevent biased analyses. The bin ranges for the histograms in Supplementary Information, Fig. S1 were selected based on percentiles (that is, to have close to the same number of data points within each bin) to avoid biased grouping.

© 2011 Macmillan Publishers Limited. All rights reserved.
Figure 1 U2OS (a-h) and HT-1080 (i-n) cells expressing promoter-truncated EGFP-paxillin in collagen gels. U2OS cells were cultured in bovine collagen gels and imaged 3-5 h after seeding. HT-1080 cells were cultured in rat-tail collagen and imaged at 18-24 h. All data were compiled from three independent experiments. All scale bars = 10 µm. (a-f) Representative fluorescence z-projections of U2OS cells with visible adhesions. Cell morphology was variable, as were protrusion dimensions, which ranged from fan-shaped (c-d, ~43% of cells) to tapered (e-f, ~32%). Approximately 25% of the cells (a-b) had multiple short protrusions. (g) Percent of fluorescent U2OS cells with at least one visible adhesion, binned according to depth in gel (i.e. distance from surface; see Methods). >50% of fluorescent cells at all assayed depths contained visible adhesions. A higher percentage of cells with visible adhesions was found near (0-67 µm) the glass surface—possibly because these areas were stiffer than areas distal to the glass. Left to right: n = 9, 13, 9, and 7 cells per bin. (h) Percent of fluorescent U2OS cells with at least one visible adhesion, binned according to the relative intensity of the protrusion background fluorescence (see Methods). A higher percentage of protrusions with intermediate background intensity had visible adhesions, possibly because high background eclipses adhesion signal whereas a very dim background indicates such low expression that adhesion intensity is also reduced. N = 10 cells for all bins. (i-l) Representative fluorescence z-projections of HT-1080 cells with visible adhesions. Cell morphology was variable, as were protrusion dimensions, which ranged from fan-shaped (i-j, ~57% of cells) to tapered (k-l, ~43%). (m) Percent of fluorescent HT-1080 cells with visible adhesions as a function of depth in the gel, presented as in (g), but showing little dependence of adhesion visibility on cell location. Left to right: n = 9, 7, 7, and 8 cells per bin. (n) Percent of fluorescent HT-1080 cells with visible adhesions as a function of the protrusion background intensity, presented as in (h) and showing a similar trend. Left to right: n = 9, 9, 8, and 9 cells per bin.
Supplementary Movie Legends

**Movie 1** A U2OS cell expressing promoter-truncated EGFP-paxillin in a 3D collagen gel about 3-5 h after seeding. The left panel shows paxillin signal; the right panel shows paxillin overlayed with the collagen reflectance image. The images are z-projections of nine z-slices. Frames were acquired every 10 s. Video plays at 10 frames/s. Corresponds to Figure 2.

**Movie 2** An HT-1080 cell expressing promoter-truncated EGFP-paxillin in a 3D collagen gel about 3-5 h after seeding. The left panel shows paxillin signal; the right panel shows paxillin overlayed with the collagen reflectance image. The images are z-projections of 20 z-slices. Frames were acquired every 10 s. Video plays at 10 frames/s.