Advanced quantification for single-cell adhesion by variable-angle TIRF nanoscopy

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ABSTRACT Over the last decades, several techniques have been developed to study cell adhesion; however, they present significant shortcomings. Such techniques mostly focus on strong adhesion related to specific protein-protein associations, such as ligand-receptor binding in focal adhesions. Therefore, weak adhesion, related to less specific or nonspecific cell-substrate interactions, are rarely addressed. Hence, we propose in this work a complete investigation of cell adhesion, from highly specific to nonspecific adhesiveness, using variable-angle total internal reflection fluorescence (vaTIRF) nanoscopy. This technique allows us to map in real time cell topography with a nanometric axial resolution, along with cell cortex refractive index. These two key parameters allow us to distinguish high and low adhesive cell-substrate contacts. Furthermore, vaTIRF provides cell-substrate binding energy, thus revealing a correlation between cell contractility and cell-substrate binding energy. Here, we highlight the quantitative measurements achieved by vaTIRF on U87MG glioma cells expressing different amounts of α5 integrins and distinct motility on fibronectin. Regarding integrin expression level, data extracted from vaTIRF measurements, such as the number and size of high adhesive contacts per cell, corroborate the adhesiveness of U87MG cells as intended. Interestingly enough, we found that cells overexpressing α5 integrins present a higher contractility and lower adhesion energy.

INTRODUCTION

Adhesion is crucial for many cellular activities. It is involved in essential physiological cell function (survival, proliferation, migration, differentiation), as well as pathological conditions (inflammation, metastasis). Cell plasma membrane and its adjacent cortex are two key components implicated in adhesion. They form a substantial meshwork whose study is critical. However, our ability to understand these important regions is limited by our ability to observe them in detail within living cells.

Cell adhesion is furthermore a multiparameter phenomenon, which can be addressed in different ways, from the nanoscale, typically on focal adhesions (FAs), to the cell scale. Numerous techniques are currently used to study adhesion at the single-cell level. Regarding all-optical approaches, super-resolution techniques have become more and more popular in recent years. These new nanoimaging techniques yielded tremendous relevant observations of focal adhesions. Single-molecule localization and stimulated emission depletion nanoscopyscopies were employed, for instance, to reveal that β3-integrin nanoclusters, appearing in FAs, are subdivided into two groups in which integrins are either active or inactive (1). Interferometric photoactivated localization microscopy allows impressive observations of FA units by providing their protein three-dimensional architecture at the nanoscale (2,3). Structured illumination microscopy combined with total internal reflection fluorescence (TIRF) microscopy has been used to reveal the growth dynamics of linear subunits which form the focal adhesion plaques (4). Moreover, single-molecule tracking can also yield interesting information about adhesion protein dynamics, such as integrins (5,6). Traction forces heterogeneity within FAs was also highlighted with a molecular fluorescent-based nanobiosensor (7). Many other techniques are commonly used to probe cell adhesion, such as traction force microscopy (8), reflection interference contrast microscopy (RICM) (9), flow chamber (10), atomic force microscopy (AFM) (11), magnetic tweezers (12), etc.

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Despite the great progress that has been made so far with these techniques and their huge forthcoming insights, most of the standard and up-to-date methods useful to study cell adhesion present a major drawback: only highly specific adhesion related to protein-protein recognition can be studied, for instance, ligand-receptor binding appearing in focal adhesion plaques. But less specific and nonspecific cell-substrate interactions also play a crucial role in cell adhesion, as various cell behaviors seem to suggest, such as during amoeboid motility, in which cells can move in the absence of FAs (13). A recent publication has shown that specific and nonspecific interplay involved in cellular adhesion appears to have a cooperative action. For example, the repulsive nonspecific force induced by the glycocalyx can alter the integrin-based focal adhesion plaques and promote cellular migration (14). Cell-substrate interactions include a lot of different nonspecific forces: polymer steric repulsion, electrostatic attraction or repulsion, polyelectrolyte-like repulsion (a mix of electrostatic and steric repulsion), van der Waals attraction, Helfrich repulsion (which gives rise to membrane undulations), or friction force (involving transmembrane proteins or lipids) mediated by the retrograde actin flow in the cell cortex. Moreover, some transmembrane proteins such as proteoglycan (e.g., syndecan, CD44) also have an ambivalent behavior. They both induce a nonspecific steric repulsion because of their long oligosaccharide chains, and their herapan sulfate (HS) side chains enable specific binding on extracellular matrix proteins such as fibronectin (15). In this case, the frontier between specific and nonspecific interactions is tenuous and so difficult to decipher. In addition to this complexity, cell-substrate adhesion can be also altered by some “external” forces, i.e., not associated to membrane components only. This “external” force can be related to cell contractility driven by myosin motors within actin filaments. To conclude, there is a large panel of specific and nonspecific forces occurring in cell adhesion, and some of these forces are still poorly studied and understood.

There is a significant difference between specific and nonspecific adhesion. Specific interactions present in FAs give rise to a strong adhesion, i.e., characterized by an important ligand-receptor binding energy (10,11,16). At the opposite, it is commonly assumed that outside FAs, cell-substrate interactions are weaker, but this was only demonstrated on biomimetic system with giant unilamellar vesicles (17) and never on living cells. Furthermore, familiar immunofluorescence pictures of FAs as linear subdiffactive structures represent only a slight fraction of the cell-substrate contact (Fig. S1). So, even though nonspecific and weak interactions are locally low or negligible, their relative weight at the cell scale could be significant. Therefore, they might have an important physiological role in cell adhesion in the same way as high specific contacts.

In this work, we propose a promising single-cell quantitative imaging technique yielding to study simultaneously regions of high and low cell adhesiveness. This method was based on a recent upgrade of variable-angle TIRF (vaTIRF) nanoscopy, as previously proposed (18). Our improved vaTIRF strategy is based on a dual measurement: the membrane height $h$ with a nanometric axial resolution (typically 10–20 nm), similar to the one achievable with other methods of nanoscopy (2,19–23), and the cell cortex refractive index, denoted $n_{cortex}$. This ($h$, $n_{cortex}$)-dual measurement provides the opportunity to distinguish strong and weak cell-substrate interplay, identifying high adhesive contacts (HACs) and low adhesive contacts (LACs) without any selective immunolabeling or fluorescent fusion proteins. Valuable information can be then extracted in real time on living cells, such as the amount, the size, and the spatial distribution of HACs and LACs and, more interestingly, their binding energies. This work was conducted using the U87MG glioma cell line, which endogenously expresses $\alpha_5\beta_1$ and $\alpha_v\beta_3/\beta_5$ integrins. U87MG cells were previously manipulated to increase or decrease the expression level of $\alpha_5$ integrin subunit to provide two subcell lines, denoted $\alpha_5^+$ and $\alpha_5^-$, respectively (24). U87MG $\alpha_5^+$ and $\alpha_5^-$ cells were used because they present a different adhesion and motility on fibronectin, as previously shown with common techniques such as cell adhesion assays and single-cell tracking (25). At first, vaTIRF investigations were conducted on $\alpha_5^+$ and $\alpha_5^-$ cells to further expand our knowledge about these cell lines. Later on, cells overexpressing $\alpha_5$ were treated either with actin drugs—a Rho-kinase inhibitor (Y27632) or activator (lyosphosphatic acid (LPA)) to suppress or improve actin-based cell contractility—or with integrin antagonists to inhibit integrin-surface binding. In addition, different analyses were performed on fixed cells to compare our vaTIRF measurements on living cells with more common ones obtained in immunofluorescence.

**MATERIALS AND METHODS**

**Cell culture**

U87MG human malignant glioma $\alpha_5^+$ and $\alpha_5^-$ cells were prepared as described in (24). $\alpha_5$ integrin subunit expression was verified by Western blot analysis and flow cytometry (25). $\alpha_5$ integrin is boosted in $\alpha_5^+$ cells and inhibited in $\alpha_5^-$ cells. At the opposite, $\alpha_v\beta_3/\beta_5$ integrin expression levels are similar in both cell line (25). U87MG cells were grown in minimum essential medium Eagle (Lonza, Bâle, Switzerland) supplemented with 10% of fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA) and 1% of antibiotics/antifungal mixture (penicillin, streptomycin, amphotericin B; Lonza) in a humidified atmosphere.
at 37°C with 5% CO₂. For vaTIRF measurements, cells were observed in a nonfluorescent culture medium at 488 nm. We used DMEM®-2 medium (Dulbecco’s modified Eagle’s medium; Evrogen, Moscow, Russia) supplemented with L-glutamine at 2 mM (Gibco), HEPES buffer at 20 mM (Gibco), and only 1% of fetal bovine serum.

Substrate preparation

To observe specific adhesion, we prepared glass surfaces coated with a thin layer of fibronectin. Thickness-corrected glass coverslips were used as substrate ([170 ± 10] μm, Assistent, Sondheim, Germany). The coverslips were firstly cleaned by immersion into freshly prepared piranha solution for 1 h (50% H₂O₂ + 50% H₂SO₄). The coverslips were then rinsed with ultrapure water and dried. Afterwards, coverslips were incubated in a solution of fibronectin (fibronectin from human plasma at 0.1%, F0895; Sigma-Aldrich, St. Louis, MO) diluted in phosphate-buffered saline (PBS) at 10 μg/mL during 1 h. Finally, to remove the nonadsorbed fibronectin, coverslips were rinsed with ultrapure water and dried again. Glass surfaces coated with RGD peptide motif were also prepared. Coverslips were firstly cleaned by immersion in the ultrasonic bath for 5 min in a water/ethanol (30:70) solution. Coverslips were then rinsed with ultrapure water and dried. Afterwards, they were activated in the ultraviolet (UV)-ozone cleaner for 5 min and were incubated 1 h with a solution of PLL-PEG-RGD at 0.15 mg/mL (PLL (20)-g-activated in the ultraviolet (UV)-ozone cleaner for 5 min and were incubated for 1 h at 37°C in the presence of 5% of CO₂. Next, vaTIRF observations were done during the first hour after incubation at 37°C.

The vaTIRF experimental setup is detailed in (18). The most significant part of our TIRF setup is a mirror mounted onto a motorized rotation stage outside the microscope. It allows us to precisely adjust the incident angle in the sample by tilting the mirror. The working range is from 0° (epifluorescence) to ~72°. Different TIRF 60° × Olympus objectives (Olympus, Tokyo, Japan) were used, with NA from 1.45 to 1.49. The calibration procedure required to establish the magnification relationship between the mirror tilting and the incident angle on the glass-water interface is detailed in a previous publication (29). As a result, the incident angle can be continuously tuned with an accuracy of ~0.1°. Two different laser excitations were used: λ = 488 and 561 nm. The laser irradiation within the sample is about a few watts per square centimeter, and the vaTIRF acquisition rate is ~1 s for the image stack. The fluorescence signal from the sample was recorded with a sensitive CMOS camera (ORCA Flash 4.0, Hamamatsu, Hamamatsu, Japan). A fine achievement of the axial focusing was provided by a z-piezo device supporting the objective (Physik Instrumente, Karlsruhe, Germany). At last, the microscope was enclosed in a hermetic controlled temperature chamber to make observations at 37°C.

The RICM setup is similar to the one proposed by J. Radler and E. Sackmann (30). We used the antiflex Zeiss objective (NA = 1.25) with red LED source of light (λ = 630 nm) and a 14-bit CCD detector (LUMO Retiga; Photometrics, Tucson, AZ). This setup enables living cells observations at 37°C.

Statistical analysis

Statistical significance was determined according to the Wilcoxon-Mann-Whitney rank test and performed with the built-in routine in IGOR Pro (Wavemetrics).

RESULTS AND DISCUSSION

High and low adhesive contact hypothesis

A typical vaTIRF measurement of a U87MG living cell adhered on glass coated with fibronectin is shown in...
Fig. 1. vaTIRF data acquisition and data processing were described in detail in a previous publication (18). Briefly, by observing only the cell membrane in contact with the substrate (through a simple plasma membrane fluorescent labeling with DiO, Fig. 1 A), we have demonstrated that a stack of 10 different TIRF images, recorded by gradually increasing the incident angle of the laser beam on the sample at the acquisition rate of ∼1 s, allows us to reconstruct the cell membrane topography of adhesive cells with a nanometric axial precision (Fig. 1 B). vaTIRF also permits us to map the effective refractive index, \( n_{\text{eff}} \), of the cell within the evanescent field. \( n_{\text{eff}} \) mainly depends on two parameters: the local refractive index of the cell cortex, \( n_{\text{cortex}} \), and the water gap thickness \( h \) between the cell membrane and the substrate. Theoretically, \( n_{\text{eff}} \) will increase together with \( n_{\text{cortex}} \) but also when \( h \) decreases, according to the asymptotic trend \( n_{\text{eff}} = n_{\text{cortex}} \) when \( h = 0 \) (18). We propose in this work a calibration, it becomes possible to compute a new image displaying \( n_{\text{cortex}} \) instead of \( n_{\text{eff}} \) (Fig. S2 C and D).

As expected, living cells do not make a flat contact on a glass substrate coated with fibronectin, and numerous tight adhesion zones appear at different places (see the dark blue regions corresponding to \( h \leq 50 \) nm on Fig. 1 B). It is well known that ligand-receptor interplay, such as integrin-fibronectin binding, significantly affects the height of the membrane in two dimensions. As a result, the membrane most closely approaches the substrate on focal adhesion zones (2,3,14,31). Furthermore, the functional unit of focal contacts includes a cluster of different proteins (integrin, paxillin, talin, FAK, actin) more or less organized in multilayers (2). This suggests a significant increase of the local refractive index in the cell cortex \( n_{\text{cortex}} \) as proposed for the first time by Izzard et al. (32,33) and Bereiter-Hahn et al. (34). Hence, focal adhesion regions should appear where the plasma membrane closely approaches the substrate and the cortex refractive index is high. These two key observations constitute the starting point of this study, paving the way to localize FAs without any specific labeling. Hence, zones in which plasma membrane is very close to the surface and the effective refractive index is high (typically \( h \leq 70 \) nm and \( n_{\text{cortex}} \geq 1.37 \)) must include highly specific cell-substrate contacts, such as focal adhesion plaques, and correspond to HAC zones. HAC zones can be located by applying a dual thresholding on both \( h \) and \( n_{\text{cortex}} \) images, as shown in Fig. 1 D. Therefore, high adhesive
contacts appear in red in this new two-color image. The rest of the cell is then supposed to include weaker adhesive contacts, LAC zones, forming the blue background in Fig. 1 D. One can also apply a single thresholding regarding the refractive index $n_{cortex}$ ($n_{cortex} \geq 1.37$) to separate HACs and LACs on the membrane-substrate distance histogram (Fig. 1 E, same color code).

To prove that $n_{eff}$ increase is due to a local protein densification and compare its spatial distribution with high $n_{eff}$ zones recorded in vaTIRF (in fact, actin filaments form very dense and large structures that exhibit a high refractive index of $\sim 1.56$ (35)). For these first investigations, vaTIRF measurements were performed on fixed U87MG $\alpha_{5+}$ cells, in which plasma membrane was labeled with DiO and F-actin with Alexa568-phalloidin (note that all other data presented in this work were strictly conducted on living cells). Firstly, it should be noted that cell fixation and membrane permeabilization damage the plasma membrane by removing many of its components (36,37). vaTIRF imaging is therefore tricky because fixed cells present many holes in their plasma membrane, as shown in Fig. S3. Nevertheless, using saponin instead of Triton X-100 for membrane permeabilization allows better vaTIRF analysis. Secondly, these membrane alterations consecutive to fixation and permeabilization lead to an important reduction of the membrane height $h$ (the mean membrane-substrate distance measured on U87MG $\alpha_{5+}$ living cells was $\langle h \rangle = 148$ nm and on fixed cells $\langle h \rangle = 77$ nm). Moreover, the nucleus becomes visible on many TIRF images (Figs. S3 and S4 A), suggesting that it is closer to the surface, like other intracellular components such as the endoplasmic reticulum or Golgi apparatus. These outcomes give rise to a global increase of $n_{eff}$ in an artificial way over the whole spreading area and so a bigger HAC surface. Despite these two drawbacks, analysis of 31 different fixed cells indicated that 42% ($\pm 12\%$) of high refractive index zones ($n_{eff} \geq 1.39$) overlap actin rich structures (stress fibers, lamellipodia), as shown in Fig. S4 E. This reasonable overlap between $n_{eff}$ and actin cytoskeleton, despite the anomalous increase of $n_{eff}$ previously mentioned, proves a certain correlation between the effective refractive index and the packing of adhesion proteins such as actin (indeed, actin is not the only component contributing to this $n_{eff}$ increase; many other proteins are also implicated in cell-substrate interactions).

Membrane height appears to be a nonrelevant parameter to distinguish adhesiveness

Typical vaTIRF investigations on U87MG $\alpha_{5+}$ and $\alpha_{5-}$ living cells in adhesion on fibronectin are given in Fig. S5. One can recognize the typical morphology of motile cells, which exhibit several close contacts on protrusive edge. A few tens of different cells were studied, and a lot of information can be extracted from such experiments. Cell-substrate contact areas were determined with TIRF images (e.g., Fig. S2, A and F). High adhesive contacts were assessed according to the dual thresholding $h \leq 70$ nm and $n_{cortex} \geq 1.37$–1.38; this last value may change a little bit between cells (e.g., Fig. S2, D and I). Histograms of membrane-substrate separation distance $h$ can be obtained from the cell topography, as well as HAC and LAC $h$-distance histograms according to the thresholding $n_{cortex} \geq 1.37$–1.38 (e.g., Fig. S2, E and J).

Fig. 2, A and B show the mean membrane-substrate distance distribution recorded at the single-cell level for both cell lines. Their asymmetric profiles exhibit a shoulder for $h = 75$ nm, more pronounced for $\alpha_{5+}$ cells, probably because of a higher number of HACs. Despite this distinction, quantitative analysis of the mean height $\langle h \rangle$ did not reveal any significative variation among $\alpha_{5+}$ and $\alpha_{5-}$ cells (Fig. 2, C–E). This means that height analysis will not provide relevant information about cell

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adhesion that would distinguish between these cell lines. This result is very important because it illustrates the benefit of the dual thresholding. This processing, which takes into account the refractive index $n_{\text{cortex}}$ in addition to the height $h$, allow us to go further and evaluate new parameters such as the number and size of HACs and their binding energy.

**High and low adhesive contacts increase with integrin expression**

TIRF measurements indicate that cell spreading area is larger for $\alpha_{5+}$ cells than $\alpha_{5-}$ cells (Fig. 3 A). Furthermore, Fig. 3, B and C indicate that this distinct spreading is not only related to highly adhesive contacts but also affects low adhesive contacts. The average HAC area plotted on Fig. 3 B confirms what has already been visible in Fig. 2, A and B: the cell line overexpressing $\alpha_5$ integrin subunit displays, as expected, a bigger HAC area per cell. It is very important to note that such high adhesive contacts only represent a small fraction of the cell/substrate spreading area: 6.8% ($\pm$ 3.2%) for $\alpha_{5+}$ cells and 2.7% ($\pm$ 2.5%) for $\alpha_{5-}$ cells. This highlights that most cell-substrate interactions are not highly specific, and at first glance, the drastic reduction of low adhesive contact area concerning $\alpha_{5-}$ cells (Fig. 3 C) means that weaker specific and nonspecific interactions are also affected by integrin expression levels.

The larger area of HACs observed for $\alpha_{5+}$ cells may have two different sources: either there is a higher number of HACs, or their size is bigger. To clarify this point, the number and the size of high adhesive contacts were assessed at the single-cell level. It appears that there are approximately three times more HACs on a single $\alpha_{5+}$ cell than on an $\alpha_{5-}$ cell (Fig. 3 D).

Regarding the size of HACs, there is no significant difference between both cell lines (Fig. 3 E). Most HAC patches are smaller than 1 $\mu m^2$, but it is not rare to find bigger ones with a size between 1 and 10 $\mu m^2$. Both results clearly reveal that HAC number per cell increases in parallel with the integrin expression level. This finding is consistent with measurements obtained with a more routine technique. The latter consists of quantifying FA plaques number by immunofluorescence on fixed cells (Fig. S1). However, our vaTIRF method offers a strong benefit; it allows us to probe focal adhesions (or, to a large extent, cell-substrate specific interactions) on living cells without any complex labeling that could perturb protein function.

**HAC and LAC binding energy decreases with integrin expression**

We have previously demonstrated that $h$-distance distributions (Fig. 2) can be used to obtain the potential energy $V(h)$ (also known as the free energy of interactions) related to cell-substrate interactions (38). This potential energy profile $V(h)$ gives an access to the binding energy $D_e$ (or dissociation energy) related to these interactions. This requires fitting the anharmonic potential $V(h)$ with the P. Morse function, thus providing $D_e$ in Joule or in $k_B T$ units ($D_e$ corresponds to the depth of the potential well, as shown in Fig. 4 A). $D_e$ represents an average of the binding energy over the cell spreading area. It is, however, possible to calculate a binding energy per unit area, denoted $D_{e_5}$ in Joules per square meter, given by $D_{e}$ divided by the HAC or LAC area per cell (Fig. 3, B and C).

Two main results appear in Fig. 4, making binding energy a key parameter to quantify the adhesion of motile cells. First of all, binding energies are always smaller for $\alpha_{5+}$ cells for any kind of cell-substrate interactions (HAC or LAC). This means that $\alpha_{5+}$ cells present a
The low adhesion energy recorded on $\alpha_{5+}$ cells must be discussed according to their migration capacity. In fact, several publications have shown that migration speed increases with $\alpha_{5\beta_1}$ integrin expression level on two-dimensional (2D) substrate and in three-dimensional microenvironment (25,39–42). We did cell tracking measurements on fibronectin, and we obtained a 2D random velocity of $\sim$7 $\mu$m/h for $\alpha_{5+}$ cells and $\sim$4 $\mu$m/h for $\alpha_{5-}$ cells. Thus, our results indicate that $\alpha_{5+}$ cells move a little bit faster in correlation with a smaller energy of adhesion. This finding corroborates previous observations on a more basic system: MDA-MB-231 cells in adhesion on fibronectin versus on poly-L-lysine (38). Cells adhered on poly-L-lysine exhibit higher adhesion strength and therefore are glued to the substrate. On the contrary, cells on fibronectin exhibiting a smaller adhesion energy can migrate. Fast migration of low adhesive cells was also revealed by the group of M. Piel under conditions of spatial confinement (13). Furthermore, the increase of cell speed along with a reduced adhesion is predicted by the mesenchymal-type migration model proposed by DiMilla et al. in the early 90s (43) and experimentally verified few years later by Palecek et al. (39). This model envisages a bell-shaped relationship between cell speed and adhesion, with a maximal speed that separates cell adhesivity in two regimes. At low adhesivity, the cytoskeleton contraction dissociates the few numbers of focal adhesions at the front and the rear of the cell, and a net cell displacement occurs. In this first regime, the cell speed increases with adhesiveness. After the maximum, the high adhesivity regime is characterized by a decrease of cell speed as adhesiveness increases. This second regime corresponds to our experimental observations, in which there are many FAs, especially at the rear of the cell. Therefore, additional bonds formed there can better withstand contractile forces mediated by the cytoskeleton. Cell translocation and speed are thus decreased with increasing adhesiveness. Live-cell observations of U87MG $\alpha_{5-}$ cells moving on fibronectin revealed the particular mesenchymal-type motility of such cells (Video S1). The U87MG $\alpha_{5+}$ cell exhibits multiple protrusive edges, which delay the cell body translocation. Before translocation, many well-defined close contacts (dark area on RICM images) appear at different cell protrusions, resulting in a high level of attachment. These contacts are maintained for a long time under contraction, thus limiting the cell displacement.

**Impact of cell contractility on binding energy**

To highlight the influence of cell contractility on the potential energy, we evaluated the $V(h)$ profile in presence of a traction force that pulls up a lipid membrane.
For this numerical simulation, we applied a traction force $F$ on a lipid bilayer, which exhibits some repeller molecules (such as glycocalyx present on the cell surface) in interaction with a glass surface (Fig. 5 A). This simulation confirms the crucial role of the additional intracellular contractile force $F$ on $V(h)$: the negative work of the traction force $(-F \times h)$ reduces the binding energy $D_e$ (44). To confirm this prediction, further experiments were performed on U87MG $\alpha_5\beta_1$ living cells treated with two different drugs acting on RhoA-associated kinase and thus on actomyosin-based cell contractility: one inhibitor (Y27632) (45) and one activator (LPA) (46). A few tens of different living cells have been studied for each molecule. Fig. S6 shows the expected effects of these drugs on actin cytoskeleton. Regarding Y27632, untreated cells exhibit a lot of stress fibers marked by bright filaments and some large lamellipodia protrusions, characterized by a more diffused signal at some edges (Fig. S6 A). After treating cells with Y27632, most stress fibers have disappeared, and sometimes an increase in lamellipodia has been observed (Fig. S6 B). On the other hand, cells to be treated with LPA were cultivated in a serum-free medium. These cells display a very low amount of stress fibers (Fig. S6 C). Therefore, under serum-free conditions, addition of LPA induced efficiently actin polymerization, and many stress fibers can be observed again (Fig. S6 D).

Results in Fig. 5, B–G confirm our prediction regarding the influence of the intracellular contractile forces. Y27632-induced reduction of cellular contractility gives rise to an increase of the binding energy $D_e$ (Fig. 5, B–D), which is due to a reduction of the traction force (Fig. 5 A). Similar results were previously obtained by traction force microscopy (47). At the opposite, the activation of RhoA signaling pathways with LPA highlights a decrease of $D_e$ (Fig. 5, E–G), connected to the presence of more myosin motors engaged in actin structures, therefore creating new contractile elements or enhancing those already existing. $V(h)$ profiles plotted in Fig. 5, B and E show that contractile force inhibition or activation is more pronounced for HACs, as confirmed by similar observations achieved with FRET-based force probe microscopy (7), in which Y27632 and LPA treatment extinguished or strengthened the local traction force appearing within or near focal adhesion plaques. However, LACs are also affected by cell contractility because myosin contractile elements are present not only in the stress fibers connected to FA but also in the cell cortex connected to the plasma membrane (48). In fact, actin cortex is characterized by its tension which can be measured by AFM or micropipette aspiration. This tension is connected to myosin activity and hindering this activity by adding an inhibitor such as Y27632 or Blebbistatin reduces the cortical tension (49,50). This result suggests that the traction forces induced by contractile elements present in the cell cortex, would be more visible in low adhesive contacts.
The outcomes of U87MG α5+ cells treated with Y27632 or LPA offer an interesting perspective to interpret binding energy variations between α5+ and α5− cells (Fig. 4). Therefore, one can suppose that the decrease of both HAC and LAC binding energies of α5+ cells is associated with a higher cell contractility. The low binding of α5− cells is thus related to a greater cell contractility, which is a hallmark of a higher motility (13). Moreover, the recent work of Chowdhury et al. provides an interesting finding regarding cell spreading and contractility (51). The authors demonstrate that cell spreading is governed by traction forces across single integrins. So, in agreement with these observations, U87MG α5+ cells exhibiting more integrins, more HACs (Fig. 3 D), and higher HAC contractility compared to α5− cells are obviously more spread out (Fig. 3 A).

**Effect of integrin antagonists on cell adhesion**

As previously described, consistent evidence suggests that HAC includes common focal adhesions. Actually, according with the overexpression of α5 integrin in U87MG cells, there is a good agreement between the mean number of HACs per cell revealed by vaTIRF (Fig. 3 D) and the mean number of focal adhesion plaques assessed by usual immunofluorescence of paxillin (Fig. S1 C). In addition to binding energy measurements and the high and low adhesiveness observed through HAC and LAC parameters, we further demonstrated that actin-based contractile elements preferentially take place in HACs (Fig. 5, B and E), in which strong integrin-fibronectin bonds are expected. However, high adhesive contacts provided by vaTIRF display a surface somewhat higher, with a different shape, than common focal adhesion units (see Figs. 1 D; S1, A and B; and S5, D and I). In fact, FA units are described as a subdiffractive linear shape, elongated along the axis of contractile force applied by the actin cytoskeleton (2,4). So, this suggests that FAs would be incorporated in HACs. In an attempt to prove this point, the highly dynamic nature of focal adhesion plaques made experiments on fixed and transfected cells quite challenging. Membrane receptors such as integrins, actin cytoskeleton, and adaptor proteins that connect actin structures to integrins within FAs (such as paxillin) do not have the same spatiotemporal dynamics. This point is crucial and explains why HAC and FA colocalization is hard to establish by tagging paxillin or vinculin, as commonly proposed to observe adhesion plaques. For instance, old focal adhesion points localized with vinculin or paxillin can subside a few minutes after adhesion disassembly. In the same way, nascent vinculin or paxillin clustering can be formed shortly before the mature focal adhesion point. A recent study clearly shows the dynamic complexity that takes place in these active zones associated with specific adhesion (7). Authors revealed a mismatch between adhesive specific contacts associated with strong pulling force and FA protein clusters of paxillin and vinculin. Dynamic observations can be performed well with vaTIRF (38), but such kinds of studies are beyond the scope of this work. To prove that integrin-based focal adhesion is included in HACs, we decided to use soluble integrin antagonists to block integrin-fibronectin binding and so FA assembly. As previously mentioned, U87MG cells exhibit on their surface α5β1 and αvβ3/β5 integrins. To inhibit integrin-fibronectin engagements (and so the related signaling pathways), two highly selective integrin antagonists were used: one specific to α5β1 integrins, called FR, and the other one specific to αvβ3 integrins, called SN (FR and SN are, respectively, components 1 and 3 in (25)). Fig. S7, A and B show the spreading of U87MG α5+ cells on fibronectin, treated with a mixture of FR and SN antagonists. As expected, cell-substrate contact area drastically decreases and even totally disappears. The small remaining contact area appears to be very stable over time. Thus, with antagonists, cells were unable to spread and move the same as usual when α5β1 and αvβ3 integrins are both engaged. vaTIRF investigations on α5+ cells treated with these two antagonists are summarized in Fig. 6. We observe that membrane height is mostly above 100 nm and the refractive index never increases; hence, no HAC events were detected. Therefore, in this case, cell adhesion only arises from low adhesive contacts. This experiment clearly demonstrates that HACs appear together with integrin-based FA, thus highlighting their mutual dependency.

More interestingly, integrin antagonists also offer an excellent opportunity to identify some adhesive components involved in LACs. Indeed, fibronectin presents many binding sites, not only for integrins (RGD, PHSRN) but also for proteoglycans (typically syndecan), through its HS binding site. Proteoglycans influence cell growth and migration, as well as actin cytoskeleton (15). They can operate as coreceptor with integrins, and they commonly associate with growth factors. For instance, syndecan-4 is well known to promote α5β1 integrin adhesion and stress fiber assembly in fibroblasts (52,53). Because of the proximity of RGD and HS binding sites on fibronectin, syndecan-4 is present close to integrin in mature FAs, but not in nascent focal adhesion plaques. Like the rest of the cells, U87MG human glioblastoma cells exhibit some proteoglycans that influence their adhesion and migration (54,55). The simplest way to reveal the presence of proteoglycans in LACs consists of observing cell spreading changes between a surface coated with fibronectin and RGD (Fig. S7). Because only integrins can bind to RGD, no more adhesion is observed on
RGD when cells are treated with a mix of FR and SN antagonists (Fig. S7, D1 and D2). The cell body is repelled far from the surface, and many filopodia appear, denoting the new spreading strategy adopted by the cell. Despite the fact that cell-substrate specific interactions are not possible on RGD with soluble antagonists, some cells seem to be able to spread a little bit on the surface, as shown in Fig. S7 D1. But time-lapse observations suggest that this dark contrast on RICM image is related to membrane undulations, probably arising from Helfrich repulsive force (56).

CONCLUSIONS

We propose in this work an advanced quantification of living cells based on vaTIRF nanoscopy. This technique allows us to measure the membrane height together with the refractive index of the cell cortex. We have confirmed that high and low adhesive cell/substrate contacts can be distinguished according to membrane height and the refractive index. On one hand, high adhesive contacts are related to specific adhesion engaging integrins. Specific adhesion is then characterized by a high binding energy per unit area $D_S$. It includes subdiffactive focal adhesion structures and larger ones, such as those associated with actin-based platforms. These structures involve cell traction forces, as usually observed by traction force microscopy. On the other hand, low adhesive contacts are associated with a low binding energy $D_S$. They are supposed to include several membrane components that support weak binding energy, such as proteoglycans, or pure nonspecific repulsive force, such as glyocalyx. Compared to other techniques used to quantify cell adhesion, vaTIRF, like traction force microscopy, appears to be a nonperturba- tive method. For instance, techniques enabling direct force measurements related to cell adhesion strength, such as AFM or flow chamber, can drastically disturb cell properties. A shear stress can induce important modification of the cell speed and contractility (47). Compared to other large field-of-view imaging techniques suited to probe cell spreading and adhesiveness, typically RICM, vaTIRF appears to be a valuable alternative. Despite the impressive recent progress of multi-wavelength RICM (22), data processing of such interferometric techniques is still complex. On the contrary, vaTIRF data analysis is easy to implement. Moreover, membrane height $h$ reconstruction with usual RICM (or multi-$\lambda$ RICM) needs a perfect knowledge of the cytoplasmic refractive index. The latter is very inhomogeneous and changes drastically over time and space. Thus, $h$-measurement with RICM is limited to cell membrane side protrusions, such as large and flat lamellipodia (22). However, the key advantage of vaTIRF is that we do not need to actually know the cytoplasmic refractive index, seeing as we measure it in addition to the membrane height. On top of that, we are currently working on a new, to our knowledge, strategy based on objective back-focal plane observation to improve $n_{cortex}$ precision (57–59). Such observation can be easily implemented and provides a fine quantification of the supercritical angle that is directly given by the refractive index of the sample.

Undeniably, a large range of data is available with vaTIRF. One can determine for both strong and weak adhesion the height of the plasma membrane, the size of the cell-substrate contact area, and the binding energy. Regarding HACs associated with strong adhesion, their
individual size and their number per cell can be assessed. We also revealed how cellular contractility can influence the binding energy. As a consequence, adhesion studies conducted on U87MG cells revealed that as expected, the number of HACs per cell increases along with integrin expression level. Moreover, as expected, the higher migration speed of U87MG cells overexpressing α5 integrins is associated with a low binding energy because of a greater contractile force. This finding was also previously observed with MDA-MB-231 cells overexpressing α5β1 integrins (41). Moreover, our study surprisingly reveals that low adhesive contacts are also affected by integrin level and play a crucial role during adhesion. This can be explained by a cross talk between integrins and Rho-GTPases, which controls cell’s contractility through myosin motors and the Arp2/3 complex (60). Actin cortex is then disturbed by integrin overexpression, as well as the plasma membrane (61).

This work opens new, to our knowledge, promising opportunities to probe integrin- or actin-mediated adhesion and migration. Further studies can be proposed when cell migration is guided along one direction according to the surface gradient of extracellular matrix proteins. This surface gradient can be obtained by electropolymerization and microfluidics (62) or by using micropatterning. Capitalizing on all the data achievable with vaTIRF, various drugs acting on focal adhesion and actin network can be tested during migration, such as integrin antagonists or contractile forces inhibitors.

SUPPORTING MATERIAL

Supporting material can be found online at https://doi.org/10.1016/j.bpr.2021.100021.

AUTHOR CONTRIBUTIONS

R.J. and D.E.A. designed research. D.E.A. and R.J. performed measurements, and R.J. supervised all experiments. D.E.A. and R.J. analyzed data. R.J. designed vaTIRF imaging setup. C.V. wrote data processing routines on IGOR Pro software. R.D. developed vaTIRF acquisition software. H.K. designed the integrins antagonist. D.E.A., C.V., M.L., H.K., M.D., and R.J. interpreted experiments. D.E.A. and R.J. wrote the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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