An integrated assay to probe endothelial glycocalyx-blood cell interactions
under flow in mechanically and biochemically well-defined environments
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Abstract

Cell-cell and cell-glycocalyx interactions under flow are important for the behaviour of circulating cells in blood and lymphatic vessels. However, such interactions are not well understood due in part to An integrated assay to probe endothelial glycocalyx-blood cell interactions

under flow in mechanically and biochemically well-defined environments

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Heather S. Davies,¹ Natalia S. Baranova^{2,2} Nouha El Amri,¹ Lilian** Our approach is demonstrated with the interaction between hyaluronan (HA, a key component of the endothelial glycocalyx) and its cell receptor CD44. We generate HA brushes in situ within a microfluidic device, and demonstrate the tuning of their physical (thickness and softness) and Heather S. Davies,¹ Natalia S. Baranova,²⁴ Nouha El Amri,¹ Liliane Coche-Guerente,³ Claude
Verdier,¹ Lionel Bureau,¹ Raif P. Richter^{24,*} and Delphine Débarrei⁵
 Chrones-iti Greation Apps. CANS Laboration contrast microscopy (RICM) and application of polymer theory. We highlight the interactions of HA brushes with CD44-displaying beads and cells under flow. Observations of CD44+ beads on a HA brush with RICM enabled the 3-dimensional trajectories to be generated, and revealed interactions in the form of stop and go phases with reduced rolling velocity and reduced distance between the bead and the HA brush, compared to uncoated beads. Combined RICM and bright-field microscopy of Sciences, *Faculty of Biological Sciences*, *School of Physics and Astronomy*. *Faculty of Minhematics and*
 $2Hbysical Science, Istibary Conference, *Stinej* Center for Stineland and *Setbary of Letes LS2 901*. *United Current addresses: Institute of Science and Technology Austria (IST Austria), An Campbell, *At of**$ *Physical Sciences, Asthury Centre for Structural Molocalary Biology, University of Leeds, Leeds LS2 9/1; United

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"Corresponding authors: <u>crichter(silenckocute)</u> Atstraction-symplectic permits of the study of the study of the study interactions are orchestrated to the beh selective blood cell-vessel wall interactions under flow. Cell-cell and cell-glycocalyx interactions under flow are important for the behandlight a blood and lymphatic vessels. However, such interactions are not well under
a lack of tools to study them in defined environments. H Our approach is demoted with the interaction between hyditional (HA, a key component of the component of the minicipality cosing of their physical (thickness and softens) and its cell: receptor CD44. We generate HA brashs mroroflude evere, and ensumption developes and softmer by the proposite (the highest on a microsos) and application of polymer theory (NECM) and application with reflection interference constrat microcopy (RICM) and appli of stop and go phases with reduced roling velocity and reduced datance between the bead
not HA brush, compared to uncoated beads. Combined RICM and bright-field microscopy of AKRI T-lymphocytes revealed complementary info **Underly** and the interation of retunating
understood due in part to
versatile *in vitro* platform
mical settings under flow,
a key component of the
prushes *in situ* within a
thenses and softness) and
the the interactio meraturions are not well understood to impart to
the mean the particulations are the particular filter, we develop a versatile in vitro platform
lefind physical and chemical settings under flow.
the sequence of the total-Entrical prinsdar and critimate suchings and critical product however hyaluronan (HA, a key component of the 44. We generate HA brushes *in situ* within a of their physical (thickness and softmess) and of their physical EVACU in Manolonia (124, a Rey component or the generate HA brushes *in situ* within a of their physical (thickness and softness) and of their physical (thickness and softness) and order memer theory. We highlight the inte Fr. We general trivents in some and wantum and soling the physical (thickness and softness) and using characterisation with reflection interference theory. We highlight the interactions of HA er flow. Observations of CD44

Keywords: Hyaluronan, CD44, glycocalyx, mechanics, biomimetics, cell-glycocalyx interactions

glycosaminoglycans (GAGs), adhesion proteins and glycoproteins that lines the luminal surface and HA receptors [5, 6], resulting in a thick HA of the blood vessel wall. It acts as the gate-keeper coat protruding into the lumen as flexible loops of cell entry to the underlying endothelium by and chains [7]. This gives rise to a HA backbone [2, 3], repulsive cushion to expel red blood cells, whilst displaying binding sites for immune cell complex self-organised film that dynamically reand stem cell rolling, adhesion and organises in response to external stimuli [8]. transmigration.

endothelial glycocalyx is the linear possesses hundreds of binding sites, although a synthesised at the plasma membrane by HA

The endothelial glycocalyx is a network of glucosamine and $\beta(1,3)$ -glucuronic acid. Several micros long [4], HA polymers are thought to be CD4⁺¹ AKN I 1-lymphocytes revealed complementary information about the Qynamics of elt folling
and cell morphology, and highlighted the formation of tehres and slings, as they interacted with a HA
broad and et flow. This brush under Itow. Iths platter mare and proportion emperator and the giveolay, and
bshould permit the study of how mechanical and biochemical factors are orchestrated to enable highly
selective blood cell-vessel wall inte Daltons, with repeating units of $\beta(1,4)$ -N-acetylanchored to the endothelium via HA synthases that provides a structural scaffold for the binding of other proteoglycans and proteins, forming a met neoty: we may may the micratorists of ration
or from Observations of CD44+ beads on a HA
prices to be generated, and revealed interactions in
velocity and reduced distance between the bead
orly information about the d r now. Ossevarators of CD+++ ceats on a rix
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the helmincal factors are orehestrated to enable highly w.

Es, biomimetics, cell-glycocalyx interactions

Daltons, with repeating units of $\beta(1,4)$ -*N*-acetyl-

glucosamine a

HA also provides binding sites for its cell receptor CD44: each polysaccharide chain fraction of these may be masked by other HA binding proteins. HA-CD44 interactions have

rolling of subsets of CD44+ T-lymphocytes [9], (a) and the firm adhesion of CD44+ neutrophils on Flow deck to inflammatory stimuli in vivo. In addition to vacuum outlet
leukocytes [9, 12-14], stem cells [15-17] and $\frac{1}{2}$ rolling of subsets of CD44+ T-lymphocytes [9],

and the firm adhesion of CD44+ neutrophilis on

the blood vessel endothelium [10, 11] in response

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to inflammatory stimuli cancer cells [16, 18, 19] employ HA-CD44
employees [9], (a) (b) (c)
the blood vessel endothelium [10, 11] in response
the blood vessel endothelium [10, 11] in response
the whole the stress of continuation with the vivit o binding for cell adhesion, as demonstrated by *in* From the blood vessel endothelial monolayers under the solution of the solution of the blood vessel endothelium [10, 11] in response
the blood vessel endothelium [10, 11] in response the solution of the blood vessel endot HA or immobilised HA/HA-expressing endothelial monolayers under static conditions or Fig. 1: Schematic depiction of the flow assay. (a) The inside flow channels, which mimic the physiological shear stress of post-capillary venules $(\sim]$ 1-4 dyn/cm² [20]). Such flow assays have also revealed that HA-CD44-mediated adhesion is tightly regulated, by mechanisms Folling of subsets of CD44 r-T-lymphocytes [9], (a) (b) (c) and

and the firm adhesion of CD44 reutrophils on

the blood vessel endothelium [10, 11] in response

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and the firm adhesion of CD44+ nutrophils on

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and the final abson of CD44+ T-lymphocytes [9], (a)

the disc due form addition for the disc due of the material relevance in the disc of the material relevance in the s stift contention in the state of the model particular is the state of the stat Cell discussion and signally cell in the signalling CD44 cell adhesion, as demonstrated by *in* $\sqrt{2}$. In the cell adhesion, as demonstrated by *in* $\sqrt{2}$. In the signal in the cell adhesion, as demonstrated by *in*

biochemical factors, there is now ample evidence that substrate mechanical properties, such as as with other cell-glycocalyx interactions studied with *in vitro* model platforms, the surfaces utilised are unlikely to reproduce key physical may somman Correct to Havan Some that a sometime control and sometime and the same sometime and the same sometime dependential monological shear stress of the scape in the same of the same of the same in the same in the sa the utilisation of immobilisation strategies in ensues and the surface state conducts are state of the section of the section of constrained ensues in the particle base are to post-capital state and incorporation of coversity simulations of post-capital state and incor most into the bandwind with the most plane that is a splet-capillary material interesting introduces are not be a splet-capillary materials and incorporation of coverslips for venuels (-14 dyn'orin' [20]). Such flow assay glycocally in films of unknown softness and $_{11}$ that the university thickness. As such, physical factors have been and the most coverage and interest the model in the searching compatible complete the including CD14 remodelling (DD1 under Momento an anulation is the tried and the searching derivating conditions [13, 22, 23], and chang their influence on cell-glycocalyx interactions meaning conditions [13, 22, 23], and changes to modal inverted microscope with RIC
activating conditions [13, 22, 23], and changes to modal inverted microscope with RIC
effectors [5, 23-26]. The wealth of information deri incores and the endine beam and the state of example with continue beam of the seal incorporation of the seal incorpo in the vector of the did in the state is the line of the state in the state in the state is the state in the state in the state is the state in the state in the state is the state in the state in the state is the state in Employed that substrate the interference contrast microscopy (RI

biochemical redevance. In addition to interference contrast microscopy (RI

biochemical redevance. In addition to interference in reflected light,

that su someonial recentions on interferior and a complement in the mathemolal light, RCM can
including this standard properties, such as surface, providing a method to characterise the
sitfiness and thickness, also profoundly in

medipolation of grycocary physical parameters
into flow assays is the lack of tools to field microscopy) with a microfluidic device. We capture, firm adhesion and rolling velocity under flow by observation of displacement along the differentiate: (i) the inherent thickness of the model surfaces; or (ii) the position of interacting

A label-free optical technique that offers sensitivity to z-directionality is reflection

parallel-plate flow chamber provides laminar flow at tuneable rates and incorporation of coverslips for functionalisation, such as gold-coated coverslips here. (b) HA brushes generated in situ on top of a SAvmonolayer on a gold surface modified with covalently attached biotin groups (bOEG/OEG monolayer). (c) Mounting of the assembled flow chamber on a multimodal inverted microscope with RICM capability (a) (b) (c) (e) $\frac{F_1 F_2}{F_3}$ a method to examine bead height with F_4 with F_5 with F_6 or F_7 and F_8 with F_9 and F_9 and F_1 and F_2 and F_3 and F_4 and F_5 and F_6 and F_7 and F_8 and respect to HA films, in addition to velocity under flow.

interference contrast microscopy (RICM). Based on interferences in reflected light, RICM can distinguish the distance between an object and a surface, providing a method to characterise the thickness of a surface layer with nanometer precision. Furthermore, the sensitivity of RICM in flow assays has been highlighted by the detection of additional arrests of particles interacting with a surface by observation of particle height with respect to the surface, compared to measurement of velocity alone [29]. Thus, it is an ideal tool to study interactions glycocalyx-mimetics and cells/cell-mimetics under flow. provides a method to examine bead height with
respect to HA films, in addition to velocity under
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surface, providing a method to characterise the

trickness of a surfac

Another factor that has hindered the with controllable chemical and physical
parameters. To this end, we integrate versatile simultations and procedures are protonuous musicle inclusions of a surface layer with anomore the specify increasing the information station of the minimal station and signalling [27, 28]. However, precision, Furthermore, Evaluation and particular in the surfact of electric in the same symmetric processor and some different microscopy is the surfact of electric microscopy in the surfact of electric software in the surface deceleration of an ⁵⁶ with the entitled are unitservent by the same in flow assays has been highlighted are unitseliked are unitsely observed to the physical perpendicular to the unitsel of physical perpendicular to the unitsel of propert surface the glycocaly parameters of the surface between the surface by observation of entirely to reproduce key physical interacting with a surface by observation of the glycocalyx. This is due in part to part the utilisa Example to the controllation of vising the compared to measurement of velocity along the contribution and surface density of Thus, it is an ideal tool to study interactions resulting in films of unknown softness and cells Here, we develop an *in vitro* flow assay to study the behaviours of circulating cells/cellmimetics on well-defined glycocalyx mimetics, controllable chemical and physical describe the tools necessary for the implementation of this methodology into a conventional inverted optical microscopy setup and illustrate the capabilities of our platform by probing HA-CD44-mediated cell-glycocalyx interactions. Specifically, we demonstrate the formation of tuneable HA based glycocalyxmimetics, including those of physiological thickness and softness, and their characterisation in situ within flow channels. In depth characterisation of the physical parameters of such films is described here with reference to polymer physics and soft matter theory. We have

RICM using 25 μ m uncoated microbeads. (a, b) Schematic representation of beads on a SAv monolayer and HA840-brush, respectively. (c) RICM images appear as fringe patterns and were taken at two (h_{meas}) . Scale bar is 5 µm. (d) Intensity profiles as a function of the distance to the pattern centre of RICM fringe patterns (black), average data (magenta) and the on SAv (h_{ref}) and HA840 (h_{meas}) from RICM images captured in red and green wavelengths ($n \ge 24$). The h_{ref} and h_{meas} include contributions from the optical surface a layer of bounds covariating attaction via
properties of the sold (see Supplementary short oligo (ethylene glycol) linkers to a provides a physical thickness termed here bead-wall wall distance measured over the typical length of time biotinylated HA (bHA) to generate HA brushes of an experiment ($n \ge 25$).

deliberately focused the main text on the principles of the setup and data analysis and provide technical details and mathematical a single or a mixture
derivations as Supplementary Information for the (as demonstrated derivations as Supplementary Information for the interested reader. In addition, we provide selected experimental examples of the insight that can be derived with this platform by demonstrating the 3D trajectories of CD44+ cell-mimetics on a HA film, and the dynamic behaviours of living CD44+ AKR1 T-lymphocytes as they roll on a HA brush under flow. With simple adaptation, $2.1.2$. Characterisation of HA brush height by this platform should incorporate alternative or further study of cell-glycocalyx interactions in microscope with RICM capability (Fig. 1c and We all *mass* incure comtourous noin use operation, but their difference of the gold (see Supplementary short oligo (ethylene glycol) linke
hromenties of the gold (see Supplementary short oligo (ethylene glycol) in
provid

2. Results and discussion
In Section (2.1) we present the details of brush
formation and characterisation using RICM and
polymer theory, with the aim to provide a
comprehensive guide to implement our
methodology Subsequ In Section (2.1) we present the details of brush formation and characterisation using RICM and polymer theory, with the aim to provide a comprehensive guide to implement our methodology. Subsequently, in Section 2.2, we highlight key applications of this platform demonstrated with selected HA brushes. The reader can refer to these somewhat independent sections as desired, in order to discover brush formation/characterisation and interaction studies with CD44+ cells/cell mimetic, respectively. 2. Results and discussion
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controllable thickness, softness and binding site density

flow chamber

fitted model (blue). (e) Measured heights (raw data) controllable flow), a spacer with defined channel properties of the gold (see Supplementary short oligo (ethylene glycol) linkers to a Information) but their difference $(= h_{\text{max}} - h_{\text{ref}})$ transparent gold film (Fig. 1a-b). Injection of Information), but their difference $(= h_{\text{meas}} - h_{\text{ref}})$ transparent gold film (Fig. 1a-b). Injection of provides a physical thickness termed here bead-wall streptavidin (SAv) in situ created a monolayer of (a) $\frac{3}{2}$ and $\frac{3}{2$ fritch model (blue), (c) Measured heights (raw data)
complex for the Thied model (blue), and HA840 (h_{mean}) from RICM images
controllable flow), a spacer with defined che
for the data green wevelengths ($n \geq 24$). The Brushes of one-end grafted HA were generated in situ within a parallel-plate flow chamber using biotinylated polymeric and oligomeric HA samples of well-defined molecular weight (M_W) , based on functionalisation protocols established previously [30, 31]. The parallel-plate flow chamber, held together under vacuum, consisted of an upper deck (with inlet and outlet for methodology. Subsequently, in Section 2.2, we
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demonstrated with selected HA brushes are
sections as desired, in order to discover brus dimensions and a coverslip displaying on its surface a layer of biotins covalently attached via reader can refer to these somewhat independent
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2.1. Formation of HA brushes wi sections as desired, in order to discover brush
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bin** formation/characterisation and interaction studies
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binding site density
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2.1.1. In situ formation of HA films within a
flo binding pockets for the further immobilisation of **2.1.** Formation of HA brushes with
controllable thickness, softness and
binding site density
2.1.1. In situ formation of HA films within a
flow chamber
 $\frac{1}{2}$.1.1. In situ formation of HA films within a
flow chamber
s (Fig. 1b). More generally, this SAv platform can be used as a 'molecular printboard' to create other cell surface/glycocalyx-mimetic films from a single or a mixture of biotinylated molecules 2.11. In sum Jornalion of HT Jimms whath a
flow chamber
Brushes of one-end grafted HA were generated in
biotinylated polymeric and oligomeric HA
samples of well-defined molecular weight (M_w) ,
based on functionalisation p Furthermore, the setup is compatible with the use of other types of functionalised surfaces (e.g., thiols on gold and silanes on glass). As such, this enables the preparation of bespoke glycocalyx and cell surface models of desired composition and complexity. chamber, held together under vacuum, consisted
of an upper deck (with inlett and outlet for
controllable flow), a spacer with defined channel
dimensions and a coverslip displaying on its
surface a layer of biotins covalen controllable flow), a spacer with defined channel
dimensions and a coverslip displaying on its
surface a layer of biotins covalently attached via
short oligo (ethylene glycol) linkers to a
transparent gold film (Fig. 1a-b) dimensions and a coverslip displaying on its
subrace a layer of biotins covalently attached via
short oligo (ethylene glycol) linkers to a
transparent gold film (Fig. 1a-b). Injection of
bioting pockets for the further im transparent gold film (Fig. 1a-b). Injection of streptavidin (SAN) *in situ* created a monolayer of biotiny lates for the further immobilisation of biotinylated HA (bHA) to generate HA brushes (Fig. 1b). More generally, t streptavidin (SAv) *in situ* created a monolayer of
biotiny pockets for the further immobilisation of
biotiny lated HA (bHA) to generate HA brushes
(Fig. 1b). More generally, this SAv platform can
be used as a 'molecular p

RICM

well-defined biochemical and mechanical Supplementary Information Section 1.1). RICM is an optical technique sensitive to the distance of

as cells or beads, as they interact with a surface.

For symmetrical objects, such as microbeads, the

distance between a bead and surface (bead-

substrate distance) can be determined

quantitatively. This was performed as cells or beads, as they interact with a surface.

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distance between a bead and surface (bead-

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quantitatively. This was performed distance between a bead and surface (beadsubstrate distance) can be determined
quantitatively. This was performed here for
precise (to within ~10 nm) characterisation of the
thickness of HA brushes.
Microbeads were injected into the flow thickness of HA brushes.

chamber and allowed to settle on the bottom \leq
surface before and after addition of bHA, thus resting either on top of the SAv monolayer or HA $\frac{1}{2}$ \frac brush, respectively (Fig. 2a-b). Images of beads the surface of the bead closest to the substrate $\frac{1}{1}$. It is the substrate in th as cells or beads, as they interact with a surface.

For symmetrical ohjects, such as microbeads, the

distance between a bead and surface (bead and surface the such as substrate distance) can be determined

quantitativel images in two wavelengths of light (red (635 nm) and green (534 nm)) enabled precise quantification of height (Supplementary as cells or beads, as they interact with a surface.

Tor symmetrical objects, such as microbeads, the

distance between a bead and surface (bead-

unnithitatively. This was performed here for precise (to within -10 mm) ch clearly illustrates that the fringe patterns on the ϵ of ϵ and the fringe patterns on the ϵ of ϵ and the distance of H_A heads the distance SAv surface and a HA brush composed of as cells or beads, as they interact with a surface.

For symmetrical objects, such as microbeads, the

distance between a bead and surface (bead-

substrate distance) can be determined and surface (bead-

substrate distan different, reflecting the different heights of the quantitive
since the Winkelses of HA brushes.

The Hard species (to within ~10 nm) characterisation of the

thickness of the Notwards.

Surface before and after addition of bHA, thus

surface before and after addition of Microbands were injected into the flow $\frac{E}{4}$ too
surface before and allowed to settle on the bottom $\frac{E}{3}$ from surface before and aller adducion of bHA, thus
restring either on top of the SAv monolayer or IIA
brus Earline the act of M₃ (1.3). Images of bead height, respectively (Fig. 2-ab). Images of bead height, respectively (Fig. 2-ab). Images of bead height preservice of the bead closest to the substrate bead height height of

Quantification of the bead-substrate distance was performed to ascertain the thickness 2.1.3. Tuning HA brush thickness were generated from the captured fringe patterns achieved by controlling the incubation conditions optical properties and the geometry of the system (Fig. 2d, Supplementary Information Section binding pockets on the SAv monolayer, which Example that which respect to the above of the SAV is the same of the SAV is the same of the SAV in which reflected in RCM, in which reflected to the basistary of a HA840 where captured in RICM, in which reflected to the beat the surface of the bead consets of the can be conset of the surface of the beat the surface of the brush (hence of the brush (Fig. 3: Tuning the density and thickness of a HA84
the surface of the bead closest to the averaging of values obtained in red and green polymeric bHA, however, cannot access all sites wavelengths was performed to obtain the due to steric hindrance imposed by bulky distance; Fig. 2e-f).

The HA brushes generated were stable and homogeneous. Following a slight reduction in HA brush thickness of \sim 5 % (Fig. 2f, $t = 13$ h) during overnight equilibration, likely due to loss the surface. At higher coverages, however, denser of either weakly bound bHA or SAv bound surface [34], surfaces showed stable thickness surface ('brush' conformation), resulting in with no significant change over the course of several days (Fig. 2f, $t = 60$ h). Thus, all subsequent experiments were performed time and/or bHA concentration, in combination following an overnight equilibration step, and could take place for several days. In addition, HA simple and precise method to tune the brush to a demonstrated by the relatively low standard deviation of the mean film thickness across measurements made throughout the flow channel

brush. HA brush thickness changes with incubation time and concentration of bHA, measured in situ with RICM. All measurements were performed on a single surface. Error bars represent standard deviations ($n \geq$ 20 beads).

beads with respect to the gold substrate. (Supplementary Information Section 1.3). affect the determination of HA brush thickness, and that photo-damage of the brush by the illuminating light is negligible over the course of an assay under the conditions used here

Ensiminal of the seale of the seale of the seale of the state in the state and green (534 mm) enabled precise surface term bars at rest energy to the state by when the control of the multion interaction in the control of the control of the sect of Cupid and the spect to the peoplementary information sect of Cupid and respect to the Qualitification of the bead-substrate (Supp and the ventral of the reference bead heigh material and the pointing internal and the geometry of the system biothly alcal called scan statues (Fig. 2d, Supplementary Information Section binding pockets on the SAv mono-
 measured on the 2nt measure of column and green polymeric than some square of a counter of the HA brush (h_{max}), followed by [34]. Larger biothylated species, such a arearging of values obtained in red and green polym s
 $\frac{200}{3}$
 $\frac{1}{2}$
 $\frac{1}{2$ Fig. 3: Tuning the density and thickness of a HA840

Fig. 3: Tuning the density and thickness of a HA840

bush. HA brush thickness changes with incubation

itme and concentration of bHA, measured in situ with

IRCM. All m biotinylated molecules can saturate all free \uparrow 0
 b \uparrow 6
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 b \uparrow 5
 b \uparrow 5
 b \uparrow 6
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 c \uparrow 6
 c \uparrow 6
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 EVCM. All measurements were performed on a single
 RICM. All measurements were performed on defines a maximal coverage corresponding to a Fig. 3: Tuning the density and thickness of a HA840

frag. 3: Tuning the density and thickness of a HA840

thush. HA brush thickness changes with incubation

fluce and concentration of bHA, measured in simple

surface. Er [34]. Larger biotinylated species, such a Fig. 3: Tuning the density and thickness of a HA840
bush. HA brush thickness changes with inculation
time and concentration of bHA, measured *in situ* with
RICM. All measurements were performed on a single
surface. Error brush. HA brush thickness changes with incubation
time and concentration of bHA, measured in situ with
RICM. All measurements were performed on a single
surface. Error bars represent standard deviations ($n \ge 20$ beads).
 time and concentration of bHA, measured *m* situ with
RICM. All measurements were performed on a single
surface. Error bars represent standard deviations ($n \geq 20$ beads).
that gravitational forces on the beads do not
af low enough surface coverages polymeric bHA adopts an unrestricted 'mushroom' conformation in which individual HA chains adopt the shape of a random coil and do not perturb one another on affect the determination of HA brush thickness,
and that photo-damage of the brush by the
illuminating light is negligible over the course of
an assay under the conditions used here
(Supplementary Information Section 1.3). packing causes repulsion of neighbouring bHA polymers, and their stretching away from the an assay under the conditions used here (Supplementary Information Section 1.3).

2.1.3. Tuning fol the thickness of HA brushes was

Tuning of the thickness of HA brushes was

achieved by controlling the incubation conditi increased HA film thickness. As demonstrated for HA840 in Fig. 3, adjustment of incubation Erizi. Tanning Enrivarias intentation conditions and Tuning of the thickness of HA brushes was achieved by controlling the incubation conditions conditionally biotinylated molecules can staturate all free binding pockets with *in situ* RICM characterisation, provided a desired thickness. Here, in order to demonstrate the structurally diverse HA brushes that can be generated with our methodology, we chose incubation conditions to yield HA840-low density (HA840-l) and high-density (HA840-h)

microbead, with multiple microbeads ($n \geq 46$) measured along the length of the flow channel. Thickness relative standard error of the mean. (e) Schematic representation of the various HA films generated, their Fig. 4: Generation of HA bushes with tuneable thickness, softmess and density of CD44 binding
Fig. 4: Generation of HA bushes with tuneable thickness, softmess and density of CD44 binding
film thickness measured by RICM o Fig. 4: Generation of HA burgher and Hannel Ha Fig. 4: Ceneration of HA brushes with tuneable thickness, some and desirty of CD44 binding sites.
Fig. 4: Generation of HA brushes with tuneable thickness, softness and density of CD44 binding sites.
This thickness measur Fig. 4: Generation of HA brankes with tuneable threaks s, sales and density of C144 branking sites. (a) HA brankes measured by RICM on various surfaces. Each point represents the bead-wall distance of a single fift m film HASB

HAdp15

HAdp15

HAdp15

HAdp15

HAdp15

HAdp15

HAdp15

HAdp15

HAdp15

HADP CD44

Hadpn:

The Sammen individual distance of a single

ed along the length of the flow channel. Thickness

were used to determine (b) t

respectively (Fig. 4a).

To produce more substantial differences
necessarily distinct limit at the method of HA surface density species of well-defined M_W was very effective. A distance between individual polymer chains, ξ shorter polymer of $M_W = 58$ kDa and oligomeric
was estimated from: HA of 15 monosaccharides were used to form HA58 and HAdp15 films, respectively. incubated under saturating conditions, to give rise film minkens meantared by Kick on various surfaces, rath point is preservant of the heavies of HA polynomic by states and the polynomic misculence of the heavies and the height of the heavier in the heavier in the heavier differentiated from that of the underlying SAv for the bending rigidity), and the monomer layer using RICM. This reflects the short contour d/magnitude meadlems Measured thickness and data derived from those measurements are shown in black
(Yer IIAdp15, ms distance, elasticity and surface density were estimated and are shown in grey. Error bus show
chiricy st For HAdp15, mms distance, elasticity and surface density were estimated and are shown
relative standard error of the mean. (e) Schematic representation of the various HA
measured thicknesss and calculated mms distance bet

polymer HA brushes provides a powerful the values of ξ were calculated as 130 \pm 18
strategy to extract other key parameters about (HA840-l), 75 \pm 10 (HA840-h) and 10.4 \pm 1.2 strategy to extract other key parameters about brush physical and chemical properties. For nm (HA58, Fig. 4b). For oligomeric HAdp15, the instance, application of polymer physics theory rms distance was estimated as 5 nm assuming it enables the distance between grafted HA chains saturates the bind
to be determined [35], and from this both the monolayer [31, 34]. to be determined [35], and from this both the

density of CD44 binding sites and the elasticity of the brush can be estimated, as described in the following sections.

From the measured brush thickness H_0 , the rms

$$
\xi^2 = \frac{8}{\pi^2} \frac{pv}{b} \left(\frac{l_c}{H_0}\right)^3
$$
 Eq. (1)

The determination of thickness of an earlier study of HA brushes [35]. In this way, with b the monomer unit length $(b = 1)$ nm, considering the basic HA disaccharide unit as the monomer) and l_c the polymer contour length calculated as $b \times (polymer M_W)/(monomer M_W)$. ach point represents the bead-wall distance of a single
point presents the bead-wall distance of a single eignt of the flow channel. Thickness
were used to determine (b) the root-mean-square (rms)
inty of CD44 binding sit are along the length or the now channel. Increases
were used to determine (b) the root-mean-square (mss)
ity of CD44 binding sites, and (d) the brush elasticity
eiver estable data are shown in black.
were estimated and ar we used to determine (t) one root-manary
stare of CD44 binding sites, and (d) the brush elasticity
eiver of CD44 binding sites, and (d) the brush elasticity
eiver estimated and are shown in grey. Error bars show
reserved effective excluded volume $v = 3.67$ nm³ (a measure for the monomer size and also the intersince the valuation of the variancy and the fillowing points (not extreme estimated and are shown in grey. Error bars show reaching points (not drawn to scale).

rading points (not drawn to scale) densities of the brush c resentation of the various HA films generated, their
rafting points (not drawn to scale).

density of CD44 binding sites and the elasticity

of the brush can be estimated, as described in the

following sections.

2.1.4. rafting points (not drawn to scale).

density of CD44 binding sites and the elasticity

of the brush can be estimated, as described in the

following sections.

2.1.4. Estimation of HA surface density

From the measured b density of CD44 binding sites and the elasticity
of the brush can be estimated, as described in the
following sections.
2.1.4. *Estimation of HA surface density*
From the measured brush thickness H_0 , the rms
distance b denotes the both CD-⁴⁴ oliming sites and the elasticity
of the brush can be estimated, as described in the following sections.

2.1.4. *Estimation of HA surface density*

From the measured brush thickness H_0 , the rms of un entired in the estimated, as described in the following sections.

2.1.4. *Estimation of HA surface density*

From the measured brush thickness H_0 , the rms

distance between individual polymer chains, ξ , was e saturates the binding pockets on the SAv 2.1.4. *Estimation of HA surface density*

From the measured brush thickness H_0 , the rms

From the measured brush thickness H_0 , the rms

distance between individual polymer chains, ξ ,

was estimated from:
 $\xi^2 =$

The density of binding sites for CD44 within the HA brush, Γ_{bs} , was estimated as:

$$
\Gamma_{\rm bs} = \frac{M_{\rm D}}{M_{\rm bs}} \xi^{-2} \qquad \text{Eq. (2)}
$$

where M_p and M_{bs} are the molecular weights of Taken together, by controlling polymer
the nolymer and the CD44 binding site (taken length and grafting density, this section has the polymer and the CD44 binding site (taken here as 5 disaccharide units, based on previous reports [6, 36, 37]), respectively. Therefore, in Eq (2) $M_{\rm p}/M_{\rm bs}$ provides the number of CD44 binding sites per HA chain, and ξ^2 accounts for the polymer brushes generated here display CD44 sites/ μ m⁻² for HA840-l to 280 \times 10³ sites/ μ m⁻² for the distribution of the sites 2.1.5. *Estimation of CD44 binding site density* the film indentation at rest by

2.1.5. *Estimation of CD44 binding site density* the film indentation at rest by

HA brush, Γ_{bs} , was estimated as:
 $\Gamma_{\text{bs}} = \frac{M_b}{$ was esumated from the fins distance of 5 finitial to be studied) [1-3]. Thus, they are potent models 1 binding site per chain. These values reflect the examination of blood cell-glycocalyx total density of binding sites on the surfaces, which for polymeric HA brushes comprises sites that are present along the entire polymer length,
demonstrate the capabilities of our platform, we not be easily accessible. The breading Fig. $\epsilon_{\text{bs}} = \frac{M_0}{M_{\text{BH}}} \xi^{-2}$ and ϵ_{th} the lift of non-interacting bead
where M_p and M_{bs} are the molecular weights of the molecular explicit the polymer and the CD44 binding site (taken le

the means to ascertain brush stiffness.
Commutation of the hand algebra (logarity line). 2.2. Probing the interactions of CD44+ Computation of the brush elastic (longitudinal) $\frac{2.2}{100}$ cells and cell-mimetics with HA films modulus, M, a measure of brush stiffness, was performed using the following equation (see Supplementary Information Sections S1.4-1.6 for 2.2.1. Development derivation and validation):

$$
M \approx \frac{13kT}{\pi \xi^2} \qquad \qquad \text{Eq. (3)}
$$

where kT is the thermal energy. As shown in Fig. 18 Pa (HA840-h) and 15.3 \pm 6.2 kPa (HA58). For of the system by confining the shape of the comparison, a 0.4 % agarose gel has an elasticity circulating cell to a simple sphere. Their receptor which for polymeric HA brushes comprises sites

that are present along the entire polymer length,

denotated the calibities of our

including those closest to the wall, which may

provide in the next section selected

not polymer, and is organised as a very thin film, its stiffness is likely to be dominated by the underlying substrate and thus in excess of 100's kPa.

more structurally complex films, such as brushes composed of HA chains of high polydispersity, or films incorporating additional molecules of the glycocalyx or cross-links, the above-described method to estimate brush stiffness cannot be

2.1.5. Estimation of CD44 binding site density the film indentation at rest by the density of binding sites for CD44 within the force of beads of varying size. The HA brush, Γ_{bs} , was estimated as:
 $\Gamma_{bs} = \frac{M_p}{M_{bs}} \xi$ Eq. (2) $-$ the lift of non-interacting beads on soft films the film indentation at rest by the gravitational force of beads of varying size. The other is based on a phenomenon called elastohydrodynamic lift under flow – which we recently reported [39].

2.1.5. *Estimation of CD44 binding site density* the film indentation at rest by the gravitational The density of binding sites for CD44 within the force of beads of varying size. The other is based hA brush, $\Gamma_{\rm{bs}}$, 2.1.5. *Estimation of CD44 binding site density* the film indentation at rest by the gravitational
The density of binding sites for CD44 within the force of beads of varying size. The other is based
HA brush, Γ_{bs} , sites/ μ m⁻² endotienal glycocalyx (which hay vary, e.g., 2.1.5. *Estimation of CD44* binding site density

The film indentation at rest by the gravitational

The density of binding sites for CD44 within the force of beads of varying size. The other is based

HA brush, Γ_{bs} 2.1.5. Estimation of CD44 binding site density the film indication at rest by the gravitational control in the system consideration of the Neuslin film indication of the Neuslin film indication of the system of a phenomen The $=\frac{V_{\text{B}}}{M_{\text{B}}}e^{-2}$ Fig. (2) was the observal to the molecular weight and M_S and M_{B} and M_{B} and M_{B} and M_{B} and V_{B} and V_{B} and V_{B} and V_{B} and V_{B} and $V_{$ binding tie densities ranging from -27×10^5 and α calculated glycocally contributed in the elastic model in t sites, μ m [1 hA840-l) to 280 × 10 ° sites/ μ m² for ^{ant} and the base of 5 min and upon inflammation, a phenomenon that remains that the system is the system of 5 min and upon inflammation, a phenomenon that remain Taken together, by controlling polymer the film indentation at rest by the gravitational
force of beads of varying size. The other is based
on a phenomenon called elastohydrodynamic lift
— the lift of non-interacting beads on soft films
under flow – which we re demonstrated how HA films can be custom-made to have desired physical (thickness, softness) and biochemical (density of binding sites) parameters using our methodology. The brushes generated here show examples ranging from thick and very soft, to thin and rigid (Fig. 4e). Furthermore, the range of accessible brush heights and elastic moduli covers the mechanical properties of the endothelial glycocalyx (which may vary, e.g., the film indentation at rest by the gravitational
force of beads of varying size. The other is based
on a phenomenon called leatsbolydrodynamic lift
- the lift of non-interacting beads on soft films
under flow – which we r the film indentation at rest by the gravitational
force of beads of varying size. The other is based
on a phenomenon called elastohydrodynamic lift
the lift of non-interacting beads on soft films
under flow – which we rece interactions under a range of physiologically relevant well-defined environments. To further the film indentation at rest by the gravitational
force of beads of varying size. The other is based
on a phenomenon called also
bothomalic lift
- the lift of non-interacting beads on soft films
under flow – which we recen provide in the next section selected experimental examples of the insights that can be obtained for interacting regimes using CD44+ cell mimetics and living cells on selected HA films. Taken together, by controlling polymer

length and grafting density, this section has

demonstrated how HA films can be custom-made

to have desired physical (thickness, softness) and

biochemical (density of binding sites length and grafting density, this section has
demonstrated how HA films can be custom-made
to have desired physical (thickness, softness) and
biochemical (density of binding sites) parameters
using our methodology. The br biochemical (density of binding sites) parameters
using our methodology. The brushes generated
here show examples ranging from thick and very
soft, to thin and rigid (Fig. 4s). Furthermore, the
range of accessible brush h

under flow

mimetics

 Eq. (3) In addition to non-interacting microbeads HAS (Fig. 4c). For HAdpl15, 40 × 10° sites/um⁻² cancelaring yievously then the season in the stational systems was estimated from the ms distance of 5 m and ¹⁰ on inflamentation, a phenomenon that remains 1 binding si 1 binding site per chain. These values reflect the two southof the proposition of blood cell-gly

total density of binding sites on the surfaces, interactions under a range of physiol

which for polymeric HA brushes compr total density of binding sites on the surfaces, in the communisor of the distribution of the surface in the surface of the rest and the component with defined environments. To further that are present along the entire pol We note that for less well-defined, or terminal end (bCD44) was incubated with SuAv Suppressionially infinited in the same in the same of the plata are phylome thus are applying that ar metrical metrical metrical metrical metrical metrical metrical metrical diation to non-interacting microbes
where kT is the thermal energy. As shown in Fig. can incorporate interacting cell-mimetics (eq. 4), the clastic $M \approx \frac{1}{\pi g^2}$ Eq. (3) in a continue the thermatic memperature metric that the therm of the continue of the control of the therm in the case of 44, the detailed metric has shown in Fig. can incorporate interacting celldescribed until now, the platform developed here can incorporate interacting cell-mimetics (e.g., functionalised microbeads) and living cells. Functionalised microbeads reduce the complexity upon inflammation, a phenomenon that remains
to be studied) [1-3]. Thus, they are potent models
for the examination of blood cell-glycocalyx
interactions under a range of physiologically
relevant well-defined environments to be studied) [1-3]. Thus, they are potent models
for the examination of blood cell-glycocalyx
interactions under a range of physiologically
interactions well-defined environments. To further
demonstrate the capabilities for the examination of blood cell-glycocalyx
interactions under a range of physiologically
relevant well-defined environments. To further
demonstrate the capabilities of our platform, we
provide in the next section selecte interactions under a range of physiologically
relevant well-defined environments. To further
demonstrate the capabilities of our platform, we
provide in the next section selected experimental
examples of the insights that available 15 µm polystyrene beads coated with SuperAvidin (SuAv, a modified form of SAv with reduced background binding) with a defined density of biotin binding sites. Here, a CD44 ectodomain construct with a biotin at the Cand living cells on selected HA films.

2.2. **Probing the interactions of CD44+**

cells and cell-mimetics with HA films

under flow

2.2.1. Development of well-defined cell-

mimetics

In addition to non-interacting micro beads to yield CD44+ beads, where the location of the biotin ascertains that the receptor orientation on the bead is comparable to the cell surface. Coverage was easily tuned by controlling the incubation concentration of bCD44, and the receptor surface density was determined by flow mentares
In addition to non-interacting microbeads
described until now, the platform developed here
can incorporate interacting cell-ministics (e.g.,
functionalised microbeads) and living cells.
Functionalised microbeads) Information Section S1.7 for details).

Fluorescence intensity (a.u.) Incubation concentration (nM)

Fig. 5: Characterisation of CD44+ beads. (a) Flow

cytometry of CD44-coated beads, after staining with

the anti-CD44 antibody IM7-FITC. (b) Median

fluorescenc cytometry of CD44-coated beads, after staining with the anti-CD44 antibody IM7-FITC. (b) Median fluorescence (left axis) and CD44 surface density (see Methods Section 4.2.1).

The beads functionalised here covered a range of
densities from ~20 to 3000 CD44 molecules per
 μ m², equivalent to 10⁴ – 10⁶ molecules per bead.
This is comparable in order of magnitude to the
number of CD44 recep densities from ~20 to 3000 CD44 molecules per μ m², equivalent to 10⁴ – 10⁶ molecules per bead. $\frac{15}{3}$ so $\frac{1}{2}$, $\frac{1}{2}$, This is comparable in order of magnitude to the number of CD44 receptors reported on macrophages [42].

under shear stress

Specific interactions between CD44-HA could be probed, as demonstrated here for CD44+ beads circles), recorded at 100 fps (shear stress 0.16 (saturated coverage) on a HAdp15 film. Videos of beads were recorded using RICM at 100 frames per second (fps) under fixed flow rates (converted to shear stress, see Methods Section and such a shear stress (σ), σ and σ a 4.2.6). Tracking procedures were developed in order to generate trajectories of beads as they moved over the HA surface (as described in the Supplementary Information Section S1.8) . This Every in a matrix of the standard correction in a matrix of the content of the matrix equivalent in the tracking from $\frac{1}{2}$ and $\frac{1$ densities from -20 to 3000 CD44 molecules per lead-wall distance. In the second vertex of magnitude to the equilibre in order of magnitude to the equilibre in the comparable in the second summer of CD44 receptors reported way 3D trajectories of single beads were eircles). In addition, the trajectory displayed
generated with high throughput. The tracking transient 'stop' and 'go' phases, with the 'stop' This is comparable in order of magnitude to the secure the electronics of CD44 receptors reported on $\frac{3}{2}$ 40^x $\frac{1}{2}$ + + + $\frac{1}{2}$ 800

monocytes [40], cancer cell lines [41] and $\frac{3}{8}$ $\frac{1}{9}$ $\frac{1}{6}$ precision is highlighted by the estimated lateral phases resolution of RICM for the beads used in this macrophages [42].

Example the interactions of CD44+ Fig. 6: Interactions of cD44+ Fig. 1.2. Examining the interactions of CD44+ can a HAAphis cell-mimetics on a HAAphis and 204 , interactions of CD44+ (able (open circle Information Section S1.9). Example the interactions between DH4HA could be (open circles). This in the interaction of the content of the content of the single CD44- (staturated coverage) on a HAdpl5 film. Videos energies interactions between the sp

As shown in Fig. 6a, the 3D trajectory of $dyn/cm²$) showed relatively constant velocity of σ ⁰¹ bead-wall \sim 60 μ m/s (closed black squares), and a bead-wall
distance of 70 nm (group block simples). This all [29], who have demonstrated with RICM the distance of \sim 70 nm (open black circles). This higher bead-wall distance for SuAv beads than uncoated beads on HAdp15 (Fig. 4) could suggest a higher surface roughness on the SuAv

film. (a) Individual 3D trajectories of a CD44- (black) and CD44+ (red) bead, showing bead-wall distance (open circles) and instantaneous velocity (closed dyn/cm2). (b) Bead-wall distance and (c) velocity histograms of all recorded time points for 8 beads (CD44- (grey) and CD44+ (red)) analysed for a selected shear stress (0.32 dyn/cm^2) at 100 fps. CD44- beads (black) and CD44+ beads (red). $n = 9-70$ beads. Error bars represent standard errors of the mean. **Example 12**
 Example 10
 Example 10 Example 12
 Example 12

coative details are streamed becomes the comparison of Average bead-wall distance (d) and velocities (e) of the surface (specific trajectorisos of beads as they become the permet to generate trajectorisos of beads. Error b a single CD44+ bead showed an overall reduced velocity of \sim 15 μ m/s (closed red squares) and lower bead-wall distance of ~20 nm (open red $(close-to-zero$ velocity) displaying reduced bead-wall distance compared to the 'go' phases. Such phases, in terms of velocity, have been observed with conventional imaging modes [43]. Here, we show that RICM provides the means to study the transient interactions in terms and CD44+ (red) bead, showing bead-wall distance
(orper cricles) and instantaneous velocity (closed
cricles), recorded at 100 fps (shear stress 0.16
cricles), recorded at 100 fps (shear stress 0.16
(CD44 - (grey) and CD44 (open circles) and instantaneous velocity (closed
circles), recorded at 100 fps (shear stress 0.16
dyn/cm²). (b) Bead-wall distance and (c) velocity
histograms of all recorded time poins for 8 beads
(CD44 - (grey) and C erciels), recorded at 100 hps (shear stress 0.16
dyn/cm²). (b) Bead-wall distance and (c) velocity
histograms of all recorded time points for 8 beads
histograms of all recorded time points for 8 beads
elected shear stre detection of interactions of surfaces bearing the intercellular adhesion molecule ICAM-1 and beads coated with anti-ICAM-1 even in the presence of a thick HA film on the surface.

observed here were emphasized by generation of The CD44-HA-mediated interactions In bright-field microscopy, the observed here were emphasized by generation of observed to roll as they translocated histogram plots of the trajectories of various HA surface under flow (F 0.32 dyn/cm^2), the bead-wall distances and snapshots shown velocities were lower for the population of little overlap. The broad distribution of velocities most likely arises through a superposition of two s). The rolling was not uniform over time and in effects: small heterogeneities in the CD44 coverage, over the surface of each single bead observed. Interestingly, as the cells interacted The CD44-HA-mediated interactions in bright-field microscopy, the
boserved here were emphasized by generation of observed to roll as they translocated
histogram plots of the trajectories of various HA surface under flow (nature of interactions involving few CD44-HA bonds. Furthermore, the average bead-wall which were particularly evident at shear stresses distance and velocity of CD44+ beads were overa physiological range of shear stresses (0-1 $dyn/cm²$, Fig. 6d-e).

Taken together, this example of the detection of CD44-HA-mediated adhesion demonstrates that our biomimetic cells and surfaces provide tools for probing physiologically relevant interactions in vitro. Furthermore, this highlights RICM as a useful detection tool, with processing bead trajectories over 1000s of frames with fast output) and similar xy sensitivity to opportunity to examine distances between the interacting bead and surface. Thus, our platform could be extrapolated to the further study of thick glycocalyx mimetics with diverse cell receptors that are required for various stages of the cell adhesion cascade, to help ascertain the role of the modulating adhesion to endothelial cell surface

CD44+ T-lymphocytes

Our assay system can also be integrated with living cells. To illustrate this, living AKR1 Tlymphocytes, transfected with CD44, were RICM and bright-field microscopy as the cells interacted with the HA surface under flow. In order to capture the cells in RICM, the bottommost surface of the cell was selected as the point rolls. Image analysis routines (implemented in of focus. In comparison to bright-field microscopy, in which the whole cell is visible, RICM is most sensitive to the part of the cell that is in direct contact with the surface.

The CD44-HA-mediated interactions In bright-field microscopy, the cells were The CD44-HA-mediated interactions In bright-field microscopy, the cells were
observed here were emphasized by generation of observed to roll as they translocated across the
histogram plots of the trajectories of various HA The CD44+ HA-mediated interactions In bright-field microscopy, the cells were
observed here were emphasized by generation of observed to roll as they translocated across the
biastogram plots of the trajectories of various The CD44-HA-mediated interactions

observed here were emphasized by generation of observed to roll as they translocated across the

bistogram plots of the trajectories of various HA surface under flow (Fig. 7a, Supplementa The CD44- HA-mediated interactions

In bright-field microscopy, the cells were

observed here were emphasized by generation of observed to roll as they translocated across the

biseds (Fig. 6b-c). At a given shear stress ota. The section of region water of the population of reade-
valid distances and snapshots shown in Fig. 7a by observation of a
subtries were lower for the population of region-of-interest (detentified by green arrow
cD44 Extry- coal computed to compute the street point in the street point in the street point in the cordinal interaction of the cell in the between the cordinal term interacting bead compared to CD44 beads. the street point in abunded of Characterius and velocity of CD44+ beads were lower in notice word in the section of containing the extractional which were particularly vident at shear stresses distance and velocity of CD44+ beads were >1 d Examere and vectors of the two states of the bottom in the same consistently reduced compared to CD44 beads respectively, Fig. 7a-b). The observ
overa physiological range of shear stresses (0-1 structures in bright-field over physiological range of shear stresses (0-1 structures in bright-field (with the focal plane at the bottom surface of the cell due to simulatneous

dyn/em², Fig. 6d-e).

Taken together, this example of the RICM/brig dyn/cm², Fig. ds4-).

The fig. df-electron surface of the electron surface of the cell due to simulate
course of the CICM/bright-field imaging) suggests that they lie detection of CD44-HA-modiated andsison relatively lo relevant interactions *in vitro*. Furthermore, this in RICM as well (Fig. 7b, Supplementary Video the high throughput data processing (capable of observed at high shear stress for other cell types
high throughput data pro highlights RICM as a useful detection tool, with 2). Similar structures have p
sign throughput data processing (capable of observed at high shear stress for
processing bead triglectories over 1000s of frames \dot{m} wire In bright-field microscopy, the cells were
d to roll as they translocated across the
face under flow (Fig. 7a, Supplementary
1-2). The rolling is evidenced in the
ts shown in Fig. 7a by observation of a
f-interest (identif observed to roll as they translocated across the HA surface under flow (Fig. 7a, Supplementary Videos 1-2). The rolling is evidenced in the snapshots shown in Fig. 7a by observation of a region-of-interest (identified by green arrow heads), whose position remains relatively stable despite substantial translocation of the cell in the direction of flow (Fig. 7a, bright-field, 30.5-32.3 In bright-field microscopy, the cells were
observed to roll as they translocated across the
HA surface under flow (Fig. 7a, Supplementary
Videos 1-2). The rolling is evidenced in the
snapshots shown in Fig. 7a by observati some occasions sliding motion could also be In bright-field microscopy, the cells were
observed to roll as they translocated across the
HA surface under flow (Fig. 7a, Supplementary
Videos 1-2). The rolling is evidenced in the
smapshots shown in Fig. 7a by observati with the HA surface, protrusions could be observed both in front of and behind the cell, In bright-field microscopy, the cells were
observed to roll as they translocated across the
HA surface under flow (Fig. 7a, Supplementary
Videos 1-2). The rolling is evidenced in the
snapshots shown in Fig. 7a by observat >1 dyn/cm2 (depicted by blue and white arrows, In bright-field microscopy, the cells were
observed to roll as they translocated across the
HA surface under flow (Fig. 7a, Supplementary
Videos 1-2). The rolling is evidenced in the
snapshots shown in Fig. 7a by observat structures in bright-field (with the focal plane at the bottom surface of the cell due to simultaneous RICM/bright-field imaging) suggests that they lie relatively close to the HA840 surface. Indeed, some protrusions were sufficiently close (up to a few µm) to the HA surface to be clearly observed in RICM as well (Fig. 7b, Supplementary Video 2). Similar structures have previously been observed at high shear stress for other cell types in vitro (e.g., via total internal reflection fluorescence microscopy), and *in vivo* (via intravital microscopy) [44, 45]. They are described as tethers, which anchor behind the cell and upon detachment project to the front of the cell thus becoming slings. Such tethers/slings have been implicated as important for immune cell trafficking, but so far have only been reported in a few instances. Indeed, their observation here is to our knowledge novel for this cell type, and may be seen in our assay due ²1 cyntrin (aceptotarior) onder and wind anitows,

energetively, Fig. 7a-b). The observation of these

structures in bright-field (with the focal plane at

the bottom surface of the cell due to simultaneous

RICM/bright espectavely, $1 \text{g}_2 \cdot n \rightarrow 0$. In the osservantor dreads structures in bright-field (with the focal plane at the bottom surface of the cell due to simultaneous the relatively is RICM/bright-field (with the coal plane at t assays the focal position likely usually lies at the cell equator, that is, at a level where the cells are in focus but tethers/slings may not be easily observed. As such, tethers/slings may be more common than previously thought, and observable in detail with the methods stated here without the need of labelling techniques. morescent imcomorphy, and in the other of the mathemore interval microscopy) [44, 45]. They are described as tethers, which anchor behind the cell and upon detachment ropict to the cont of the cell thus becoming slings. S

The simultaneous acquisition of images in bright-field and RICM provides diverse information about the cell's architecture as it ImageJ) provided the means to ascertain cell velocity, area and roundness, calculated as $4/\pi \times$ Area/(Major axis)², from bright-field images, and contact area size and roundness from RICM images, as demonstrated for a single cell

Images in bright-field (left) and RICM (right) as a cell rolled on the surface, under an imposed shear stress of 1.1 dyn/cm2 ; a series of snapshots at selected times (indicated) is shown. Green arrows demonstrate a region-ofslings, respectively. The bright-field image also shows a weaker 'ghost' image of the cell due to parasite d, red triangles), and velocity (from bright-field; e, black circles) under increasing shear stress (e, dashed line). Scale bar is $10 \mu m$. 37.2.

31.7a

31.7a

31.7a

31.8a

31.8a

31.8a

31.8a

31.8a

31.8a

31.8a

31.8a

32.8a

32.8 **18.** $\frac{1}{2}$ **18.** $\frac{1}{2$ defined to the surface under the surface **Example 19**
 Example 120
 Example 120
 Example 120
 Example 120
 Example 11.3
 Example 120
 Example 11.3
 Example 120
 Example 120
 Example 120
 Example 120
 Example 120
 Example 120
 Example 1

contact area shape as the cell rolled, these data also highlight the much smaller area of contact overall cell size (Fig. 7c). Furthermore, while the 7d, black), the contact interface was less regular changes to the deformation of the contact area rather than the whole cell shape as the cell rolled. Further insight was obtainable by observation of cell velocity. For instance, during spikes in cell coincided with tether detachment (Fig. 7a, Supplementary Video 1), cell area and contact suggest transient decompression of the flattened cell as it rolled faster, or heterogeneities in cell shape along its circumference, which were
emphasized as it rolled. 3. Conclusion emphasized as it rolled. ow (Fig. 7a, Supplementary Video 1). As dynamics of cell rolling, size, contact
inghilighting the fluctuating cell shope and deformation as cells interact with a sur-
inglahight the much smaller area of contact biomimetic

well as highlighting the fluctuating cell shape and deformation as cells interact with a surface under contact area shape as the cell rolled, these data flow. In addition, this example illustrates how between the cell and HA surface compared to the cells. This approach has the potential to be in shape (Fig. 7d, red), suggesting greater circulating cells on real glycocalyces. In this way, changes to the deformation of the contact area our integrated assay can encompass a large range sling, respectively. The bright-field image also shows a weaker "gloost weaker "also a for cell due to parasie
effections. (b) At an imposed shear stress of 1.6 dyn/cm², another cell has a long sling, observable in both area transiently decreased. Such observations the real endothelial glycocalyx-blood cell
suggest transient decompression of the flattened interactions more closely (thus facilitating tests reveals complementary insight regarding the dynamics of cell rolling, size, contact area and biomimetic surfaces can be interfaced with living **Example 19**
 COVECITY:
 COVECIT 199
 COVE extended further, to observe circulating cell- $\frac{8}{6}$ of $\frac{1}{100}$ $\frac{1}{20}$ $\frac{1}{10}$ $\frac{1}{20}$ $\frac{1}{10}$ $\frac{1}{20}$ $\frac{1}{10}$ $\frac{1}{20}$ or $\frac{1}{20}$ or $\frac{1}{20}$ is shown. Green arrows demonstrate a region-of-rolling. White and blue arrows point to teth endothelial monolayers [46], and even to live circulating cells on real glycocalyces. In this way, of CD44+ T-lymphocytes on a HA840-h brush. (a)
ed on the surface, under an imposed shear stress of 1.1
rolling. White and blue arrows point to tethers and
pishown. Green arrows adenosinate a region-of-
rolling. White and b of complexity, from fully reconstituted models that are well-defined and capture selected aspects of the cell-glycocalyx interaction in their pure form (thus facilitating mechanistic studies and the identification of new phenomena), to fully cellular systems that reproduce the complexity of the real endothelial glycocalyx-blood cell It many transies) and roundness of contact area (trom KLM);
circles) under increasing shear stress (e, dashed line).

reveals complementary insight regarding the

dynamics of cell rolling, size, contact area and

deformati of biological relevance). reveals complementary insight regarding the
dynamics of cell rolling, size, contact area and
deformation as cells interact with a surface under
flow. In addition, this example illustrates how
biomimetic surfaces can be int detormation as cells interact with a surtace under
flow. In addition, this example illustrates how
biomimetic surfaces can be interfaced with living
cells. This approach has the potential to be
extended further, to observe

simultaneous imaging in bright-field and RICM
integrated methodology to shed light on cell-The assay system reported here represents a new,

glycocalyx interactions under flow in and coated with a 5 nm layer of gold [30], to mechanically and biochemically well-defined yield a surface that remains transparent (for glycocalyx-mimetics in 3D offers the opportunity to study the importance of physical parameters of the endothelial glycocalyx. Here, we have reported on the development of model HA surfaces with tuneable physical and chemical optimal seal of the laminar flow chamber. Oligoproperties and demonstrated the wealth of insight that they can provide on cell adhesion under flow OEG (bOEG) thiol were purchased from with two selected examples. Such factors are inherent to physiological glycocalyces, and are likely to be modulated during inflammation. Therefore, this insight could be crucial to our understanding of how changes in inflammation enzymatic synthesis [48], were purchased from can govern controlled cell entry to the Hyalose, OK, USA. The automated solid-phase endothelium. This could in the future be tackled synthesis of the HAdp15 oligosaccharide, and glycocalyx interactions under flow in and coated with a 5 nm layer of gold [30], to
mechanically and biochemically well-defined yield a surface that remains transparent (for
emironments. The ability to track cells or cell by comparison of cell adhesion behaviours across different HA brushes. Furthermore, while we 4.2 . Methods have utilised HA-CD44-mediated cell-glycocalyx interactions to demonstrate our methodology $4.2.1$. $CD44+$ here, this platform also provides room for diversity in glycocalyx-mimetics, allowing for co-presentation of glycocalyx components in various concentrations, combinations and Functionalisation with bCD44 was performed at architectures to untangle how biochemical and mechanical cues synergistically control the selective and demonstrate of the selective and minimal of the selective and minimal of the signature of the station blood vessel wall. that they can provide on eel adhesion under flow. OEG (bOEG) thicl

with two selected examples. Such factors are Polypure, Oslo, Norway. Su

inherent to physiological glycocalyces, and are from Sigma Aldrich. Reducing

li inherent to physiological glycocalyces, and are from Sigma Aldrich. Reducing-

likely to be modulated during inflammation. HA (bHA) with molecular veright

likely to be modulated during inflammation and 58 ± 3 kDa, prepar IRey to be monduated unrely intrammation. HA (1914) With molecular weigns 8+1 = 00 kDa

Therefore, this insight could be crucial to our and 58 ± 3 kDa, prepared by in vitro ehemo-

understanding of how changes in inflamma

Experiments with cell- and surface-biomimetics a thermomixer (Eppendorf) in the dark. Beads
were performed with HEPES buffer, pH 7.4: 10 were weaked 5 times and 10000 events analysed mM HEPES (Fisher Scientific, Illkirch, France), with a BD LSR II Analyzer Flow Cytometer with 150 mM NaCl (Sigma Aldrich, Saint-Quentin DIVA 6.3.1 software (BD Biosciences). Error Fallavier, France), 2 mM CaCl₂ (Sigma Aldrich)

(SuAv)-functionalised polystyrene microbeads were purchased from Polysciences Europe GmbH mete, ms pattom also provides from for *cancelerisation*
diversity in glyocoalyx-mimetics, allowing for SuAv-beads were washed in HEPES
co-presentation of glyocoalyx components in centrifugation at 12000 \times g for
variou human extracellular domain of CD44 (residues 1- 4.2.2. Surface preparation ex situ 267) and a C-terminal biotin tag (bCD44) was generated as described previously [47]. The monoclonal anti-CD44 antibody IM7 conjugated (Saint Quentin Yvelines Cedex, France).

glycocalyx interactions under flow in and coated with a 5 nm layer of gold [30], to mechanically and biochemically well-defined yield a surface that remains transparent (for environments. The ability to track cells or cell mimetics interactions with well-defined properties of gold (required for thiol-monolayer and coated with a 5 nm layer of gold [30], to
yield a surface that remains transparent (for
optical microscopy) yet displays the chemical
properties of gold (required for thiol-monolayer
formation). The gold-coating was pe and coated with a 5 nm layer of gold [30], to
yield a surface that remains transparent (for
optical microscopy) yet displays the chemical
properties of gold (required for thiol-monolayer
formation). The gold-coating was pe and coated with a 5 nm layer of gold [30], to
yield a surface that remains transparent (for
optical microscopy) yet displays the chemical
properties of gold (required for thiol-monolayer
formation). The gold-coating was pe formation). The gold-coating was performed using a mask to ensure that only the central part of the surface was coated with gold, while the outer parts were retained bare to guarantee an and coated with a 5 nm layer of gold [30], to
yield a surface that remains transparent (for
optical microscopy) yet displays the chemical
properties of gold (required for thiol-monolayer
formation). The gold-coating was p ethylene glycol (OEG) thiol and biotinylated and coated with a 5 nm layer of gold [30], to
yield a surface that remains transparent (for
optical microscopy) yet displays the chemical
properties of gold (required for thiol-monolayer
ormation). The gold-coating was per Polypure, Oslo, Norway. SAv was purchased from Sigma Aldrich. Reducing-end biotinylated HA (bHA) with molecular weights 840 ± 60 kDa and 58 ± 3 kDa, prepared by in vitro chemoand coated with a 5 nm layer of gold [30], to
yield a surface that remains transparent (for
poptical microscopy) yet displays the chemical
properties of gold (required for thiol-monolayer
formation). The gold-coating was and coated with a 5 nm layer of gold [30], to
yield a surface that remains transparent (for
optical microscopy) yet displays the chemical
properties of gold (required for thiol-monolayer
formation). The gold-coating was p and coated with a 5 nm layer of gold [30], to
yield a surface that remains transparent (for
optical microscopy) yet displays the chemical
properties of gold (required for thiol-monolayer
formation). The gold-coating was pe conjugation with biotin at the reducing end, has been described in [34]. and coated with a 5 nm layer of gold [30], to
yield a surface that remains transparent (for
optical microscopy) yet displays the chemical
properties of gold (required for thiol-monolayer
formation). The gold-coating was p and social many of the social many of the prince with a surface that remains transparent (for optical microscopy) yet displays the chemical formionolayer properties of gold (required for thiol-monolayer formation). The go Example 1.1 The Solution consideration with the surface was coated with gold, while the outre parts were retained bare to guarantee an optimal scal of the laminar flow chamber. Oligo-
ethylene glycol (OEG) thiol and bioti contributed in a thermomic worser control of the laminar flow chamber. Oligo-
ethylene glycol (OEG) thiol and biotinylated from
DoBG (bOEG) thiol and biotinylated
DOEG (bOEG) thiol and biotinylated
from Sigma Aldrich. Red

characterisation

SuAv-beads were washed in HEPES buffer by centrifugation at $12000 \times g$ for 5 min. a working volume of 50 µL and bead concentration of 0.5 % (w/v) for 1 hour at 22 °C 1000 rpm. CD44+ beads were then washed, as described above. Beads were stored at 4° C, and used for flow assays within one week. HA (bHA) with molecular weights 840 ± 60 kDa
HA (bHA) with molecular weights 840 ± 60 kDa
and 58 ± 3 kDa, prepared by in vitro chemo-
enzymiatic synthesis [48], were purchased from
Hyalose, OK, USA. The automated solid-ph Entricution of S. USA. The unitarized with a beat the symber of a standard solution synthesis [48], were purchased from Hyalose, OK, USA. The unitarial solution synthesis of the HAdp15 oligosaccharide, and conjugation wit

and 1 mM NaN₃.
Unfunctionalised and SuperAvidin $Median \downarrow \times 1.4826$ The value 1.4826 adjusts the meratons to entromosize our memotology 4.21 , $L1p44+$ cell-
nectrosition of entromosize and the memotology 4.21 , $L1p44+$ cell-
diversity in glycocaly-mimetics, allowing for Sukv-beads were washed in HEPES buffer by
d meannant cless synergistering control the concentration of 0.5 % (Wv) for 1 hour at 22

blood vessel wall.
 4. Materials and methods described above. Beads were strong and migration of cells at the with agitation in a t Seet wall.

See the threads were shown by the reads were shown that the track start
 Materials and methods described above. Beads were stored at ⁴°C, and
 Materials Materials in the vasally in the vast were stored **4. Materials and methods**

used for flow seasys within on week.
 4.1. Materials

Experiments with cell-

and surface-biomimetics (For flow sytometry, CD44+ beads were

performed with HEPES burfer, pH 7.4:10 were vashed 35 mm of the same state of the same were stated to the same were propored with MA-IITC for 1 hat 1400 pm in the same proon at the same state of the same proportion with the same proportion with HEPES (Fisher Scientific, I For flow cytometry, CD44+ beads were were washed 5 times and 10000 events analysed Enginesian of the HAdple Summatrical conditions (Fig. 1.126 per parameter and expandent with biomit at the reducing end, has been described in [34].
 4.2. Methods the HAdpl5 oligosaccharide, and the nearched in [34].
 Fyatoe, or, ebox¹ in a taximated solar place, experimentation synthesis of the HAdp15 oligosaccharide, and conjugation with biotin at the reducing end, has been described in [34].
 4.2. Methods
 α *Safax*-beads wer bars of flow cytometry data represent robust $Median_x$ } × 1.4826. The value 1.4826 adjusts the robust value to the equivalent of a normal population distribution. rSD values were calculated in BD FACSDiva Software. SuAv-beads were washed in HEPES buffer by
centrifugation at 12000 × g for 5 min.
Functionalisation with bCD44 was performed at
a working volume of 50 µL and bead
concentration of 0.5 % (w/v) for 1 hour at 22 °C
with agita used for flow assays within one week.

For flow cytometry, CD44+ beads were

incubated with IM7-FITC for 1 h at 1400 rpm in

a thermomixer (Eppendorf) in the dark. Beads

with a BD LSR II Analyzer Flow Cytometrs analysed

Gold-coated coverslips were exposed to UV/ozone (Jelight, Irvine, CA, USA) for 10 min and submerged in a thiol mixture composed of bOEG and OEG in a 2:98 molar ratio at a final thiol concentration of 1 mM in ethanol within a glass container. The submerged coverslip was stored at 4° C for a minimum of 18 h and

assembly in the parallel-plate flow chamber, the coverslip was washed with ethanol and dried glucose Dulbecco's modified eagle's medium

The circular parallel-plate flow chamber (GlycoTech, MD, USA) was fitted with inlet/outlet tubing (PTFE, with internal diameter 4.2.5. Multi-modal 0.56 mm, Fisher Scientific) and a rubber gasket (spacer, with defined channel of dimensions: $0.250 \times 2.5 \times 20$ mm $(h \times w \times l)$). Prior to formation of HA brushes, the chamber was assembled with a blank 35 mm coverslip, held assembly in the parallel-plate flow chamber, the passaged twice a week and cultured in high
coverslip was washed with ethanol and dried glucose Dublecco's modified eagle's medium
under N₂. Functionalisation of HA films bovine serum albumin (BSA), as detailed below. To account for the dead volume of ~ 50 µL, passivation and functionalisation steps were preceded by injection at 100 µL/min for 1 min. Flow rates were controlled by a syringe-pump (KDS Legato 110). coverslip was washed with ethanol and dried gluoose Dubecoco's modified eagle's medium puredicted in the state inactivated fetal bovine serum (FBS) and $4.2.3$. Functionalisation of HA films in situ periculiar (100 U/mL) 4.2.3. Functionalisation of HA films in situ

hencimicatived fetal bowin perallel-plate flow chamber

within parallel-plate (100 U/mL)/streptomycni (100

The circular parallel-plate flow chamber

(Fisher Scientific) at 37 charge (PTFE, with internal diameter 4.2.5. Multi-modal imaging and data
charge intervoulet tubing (PTFE, with internal diameter 4.2.5. Multi-modal imaging and data
of some resistent of all a rubber gasket acquisition
for

BSA passivation was performed with 10 mg/mL BSA in HEPES buffer for 15 minutes at followed by washing in ultrapure water for 25

ex situ with bOEG/OEG (see above) was Beads were injected onto HA films and left to incorporated into the BSA-passivated flow was washed with HEPES buffer at 50 µL/min for 10 min before functionalisation. Following equation [39]: Functionalisation steps were performed at a wallformation of HA brushes, the chamber was modalities. We provide through details abuse
sombled with a blank 35 mm coverslip, held this conversion, in Supplementary Information, between the
determination of HA brushes, the rate of 3.9 μ L/min, Eq. (3)), in order to maintain the same wall shear stress (and thus the same solution viscosity ~ 1 mPa.s at room temperature mass transport conditions) as used in QCM-D Experimentation of functionalisation steps were and when the same when the solar steps and the second by microionalisation of microbe preceded by injection at 100 μ L/min for 1 min. explured in two wevelengths. RICM imp experiments, which have previously been used to microbeads were performed at a temperature of develop and characterise the surface $24\degree C$. Tracking are achieve of the hards in three preseded by injection and candidation step with the method in the strategy incredict by injection at 100 $\mu L/m$ for 1 min. captured in two wavelengths. RICM videos were Flow rates were controlled by a syring-pump captured inicionalisation strategy [50, 51]. SAV was
injected at a concentration of 20 μ g/mL for 30
Information Section S1.8 minutes, and the surface washed with HEPES buffer for 10 min. For HA films used in Fig. 4, 4.2.7. Flow assay with $CD44 +$ cells addition of bHA was performed as follows: HAdp15, 1 μ g/mL for 35 min; HA58, 1 μ g/mL temperature, on a monolayer of a modified-form
for 90 min: HA840-h 20 μ g/mL for 105 min: of SAv, Traptavidin, which has slightly reduced for 90 min; HA840-h, 20 µg/mL for 105 min; However of 3.9 μ Jmin for the sequential incubation is the sequential in the sequential in the sequential included oversity functionalised by with the sequential incubation incorporated into the BSA-passivated flow sett The motion of the steps shown in the surface of the steps shown in Fig. 3. The surface of Sole above) was been shown in the producted and CD44-
incorporated into the BSA-passivated flow steps for Smin, and videos were the in HEPES buffer for 10 min and left overnight to sample stage of the microscope was placed inside
equilibrate in the absence of flow. a box for control of temperature and set to 37 °C Example and solutions in the surface in the dimension of Halmatic and kinds were injected on the Halmatic surface in the surface of the system of μ , was was well and the methanic of the BA-passivated flow settle for 5 chamber, and scaled under vacuum. The surface

was washed with HEPES buffer at 50 µL/min for was converted to shear stress, r, 10

10 min before therefored at a wall-

flucture fixed flow rates, r, 10

4.0.0.25 dyn/cm? (e 10

In the fore

in the particonalisation steps were performed at a wall-

Functionalisation steps were performed at a stull-

calculation $t = \frac{69\eta}{h^2w}$. Eq. (4)

shear stress of 0.0025 dyn/cm² (qual to a flow

the

AKR1 T-lymphocytes, naturally CD44-, working solution was exeminged for setain free ransiected with $CD44+$ were originally antifungal agent Amphotericin B (Sigma
generated and kindly provided by Robert Hyman Aldrich). Cells were injected on the HA840-h (SALK Institute, La Jolla, CA, USA). Cells were

assembly in the parallel-plate flow chamber, the passaged twice a week and cultured in high coverslip was washed with ethanol and dried glucose Dulbeco's modified eagle's medium (DMEM, Sigma Aldrich) supplemented with $4.$ 4.2.5. Punctionalisation of HA Jums in stide
within parallel-plate flow chamber
The simple parallel plate flow showber
(Fisher Scientific) at 37 °C, 5 % CO₂ in T75 passaged twice a week and cultured in high passaged twice a week and cultured in high
glucose Dulbecco's modified eagle's medium
(DMEM, Sigma Aldrich) supplemented with
heat-inactivated fetal bovine serum (FBS) and
penicillin (100 U/mL)/streptomycin (100 µg/mL)
(Fi (DMEM, Sigma Aldrich) supplemented with heat-inactivated fetal bovine serum (FBS) and passaged twice a week and cultured in high
glucose Dulbecco's modified eagle's medium
(DMEM, Sigma Aldrich) supplemented with
heat-inactivated fetal bovine serum (FBS) and
penicillin (100 U/mL)/streptomycin (100 μ g/mL) passaged twice a week and cultured in high
glucose Dulbecco's modified eagle's medium
(DMEM, Sigma Aldrich) supplemented with
heat-inactivated fetal bovine serum (FBS) and
penicillin (100 U/mL)/streptomycin (100 µg/mL)
(F flasks (Fisher Scientific). passaged twice a week and cultured in high
glucose Dulbecco's modified eagle's medium
(DMEM, Sigma Aldrich) supplemented with
heat-inactivated fetal bovine serum (FBS) and
penicillin (100 U/mL)/streptomycin (100 µg/mL)
(Fi

acquisition

A commercial optical microscope was adapted to incorporate bright-field and RICM imaging modalities. We provide thorough details about this conversion, in Supplementary Information, Section S1.1, in order for other laboratories to follow this procedure should they desire. mean tware in the state of the state of the state of the state of the RICM and computed relation of Tisher Scientific).

4.2.5. *Multi-modal* imaging and data acquisition

4.2.5. *Multi-modal* imaging and data acquisition It sust Socialities and a 37 example and additional distribution of a 37 example flasks (Fisher Scientific).

4.2.5. Multi-modal imaging and data acquisition A commercial optical microscope was adapted to incorporate brig A commercial optical microscope was adapted to
incorporate bright-field and RICM imaging
modalities. We provide thorough details about
this conversion, in Supplementary Information,
Section S1.1, in order for other labora

For data acquisition of microbeads at rest and where lift was observed, RICM images were captured in two wavelengths. RICM videos were captured using an exposure time of 1.1 ms and frame rates as described in the main text.

For data acquisition with CD44+ AKR1 T-cells, dual bright-field and RICM images were captured with an exposure time of 30 ms and frame rate of 10 fps.

The gold-coated coverslip functionalised $\frac{4.2.6}{\text{body}}$ Flow assay with uncoated and CD44+ beads

e chamber was modalities. We provide thorough details about
coverslip, held this conversion, in Supplementary Information,
passivated with Section S1.1, in order for other laboratories to
detailed below. follow this proce settle for 5 min, and videos were then recorded in RICM under fixed flow rates. The flow rate, Q, was converted to shear stress, τ , by applying the

$$
\tau = \frac{6Q\eta}{h^2 w} \qquad \qquad \text{Eq. (4)}
$$

with w and h the channel dimensions, and η the Section S1.1, in order for other laboratories to
follow this procedure should they desire.
For data acquisition of microbeads at rest
and where lift was observed, RICM videos were
captured in two wavelengths. RICM videos and 0.7 mPa.s at 37 °C). Measurements with For data acquisition of microbeads at rest
and where lift was observed, RICM images were
captured in two wavelengths. RICM videos were
captured using an exposure time of 1.1 ms and
frame rates as described in the main tex 24 °C. Tracking procedures of the beads in three captured in two wavelengths. RICM videos were
captured using an exposure time of 1.1 ms and
frame rates as described in the main text.
For data acquisition with CD44+ AKR1
T-cells, dual bright-field and RICM images were
a Information Section S1.8. For data acquisition with CD44+ AKR1
T-cells, dual bright-field and RICM images were
captured with an exposure time of 30 ms and
drame rate of 10 fps.
 $4.2.6$ Flow assay with uncoated and CD44+
beads
Beads were injected o

A HA840-h brush was formed at room temperature, on a monolayer of a modified-form and warrel and exposed time of 30 ins and
frame rate of 10 fps.
4.2.6. *Flow assay with uncoated and CD44+*
beads
Beads were injected onto HA films and left to
settle for 5 min, and videos were then recorded in
RICM under Fig. 6. Flow assay with uncoated and CD44+
beads
Beads were injected onto HA films and left to
settle for 5 min, and videos were then recorded in
RICM under fixed flow rates. The flow rate, Q ,
was converted to shear str stability of HA brushes at 37 °C here [49]. The sample stage of the microscope was placed inside Beads were injected onto HA films and left to
settle for 5 min, and videos were then recorded in
RICM under fixed flow rates. The flow rate, Q ,
was converted to shear stress, r, by applying the
following equation [39]:
 for flow assay experiments with cells. The working solution was exchanged for serum-free was converted to shear stress, r, by applying the following equation [39]:
 $\tau = \frac{6Q\eta}{\hbar^2 w}$ Eq. (4)

with w and h the channel dimensions, and η the

solution viscosity (~ 1 mPas. at room temperature

supplement following equation [39]:
 $\tau = \frac{6\varrho\eta}{\hbar^2\pi v}$ Eq. (4)

with w and h the channel dimensions, and η the

solution viscosity (~1 mPa.s at room temperature

and 0.7 mPa.s at 37 °C). Measurements with

microbeads were $\tau = \frac{6q\eta}{hq_w}$ Eq. (4)
with w and h the channel dimensions, and η the
solution viscosity (~1 mPa.s at room temperature
amicrobeads were performed at a temperature of
microbeads were performed at a temperature of
 24

surface under an imposed shear stress of 0.2 2. O'Callaghan, R., Job, K.M., Dull, R.O., and
dyn/cm² and videos were recorded Hlady, V., Am J Physiol Lung Cell Mol
simultaneously in bright-field and RICM (60× oil Physiol $dyn/cm²$ and videos were recorded surface under an imposed shear stress of 0.2 2. O'Callaghan, R., Job, K.M., Dull, R.O., and
dyn/cm² and videos were recorded Hlady, V., Am J Physiol Lung Cell Mol
simultaneously in bright-field and RICM (60× oil Physiol objective). When cells were observed in the field surface under an imposed shear stress of 0.2 2. O'Callaghan, R., Job, K.M., Dull, R.O., and

simultaneously in bright-field and RICM (60x oil
 P_{h} sion, 2011. **301**(3): p. L353-60.

objective). When cells were observed surface under an imposed shear stress of 0.2 2. O'Callaghan, R., Job, K.M., Dull, R.O., and
dyn/cm² and videos were recorded Hlady, V., $Am \ J Physiol Lung Cell Mol
simultaneously in bright-field and RICM (60× oil *Physiol*, 2011. **301**(3): p. L353-60.
objective). When cells were observed in the field 3. Mark,$

under an imposed shear stress of 0.2 2. O'Callaghan, R., Job, K.M., Dull, R.O., and

and videos were recorded Hlady, V., Am J Physiol Lung Cell Mo.

ecously in bright-field and RICM (60x oil Physiol, 2011. 301(3): p. 1.35 surface under an imposed shear stress of 0.2 2. O'Callaghan, R., Job, K.M., Dull, R.O., and
dyn/cm² and videos were recorded Hlady, V., *Am J Physiol Lung Cell Mol*
simultaneously in bright-field and RICM (60x oil *- Ph* background was generated by performing a zproject average intensity across the full video). 6. surface under an imposed shear stress of 0.2 2. O'Callaghan, R., Job, K.M., Dull, dyn/cm² and videos were recorded Hlady, V., *Am J Physiol Lung* imulteneously in bright-field and RICM (60x oil *Physiol, 2011.30133-60*, roundness from bright-field images, cells were segregated from the background by applying a surface under an imposed shear stress of 0.2 2. O'Callaghan, R., Job, K.M., Dull, R.O., and

simultaneously in bright-field and RICM (60× oil *Fhysiol Lung Cell Mol*

simultaneously in bright-field and RICM (60× oil *Fhys* surface under an imposed shear stress of 0.2 2. O'Callaghan, R., Job, K.M., Dull, R.O., and

simultaneously in bright-field and RICM (60x oil

simultaneously in bright-field and RICM (60x oil

solutive). When cells were o surface under an imposed shear stress of 0.2 2. O'Callaghan, R., Job, K.M., Dull, R
dyn'om² and videos were recorded Hady, V., *Am J Physiol Lung C*
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An integrated assay to probe endothelial glycocalyx-blood cell interactions under flow in mechanically and biochemically well-defined environments

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S1. Supplementary Information

S1.1 Reflection interference contrast microscope setup

This section provides all the necessary details to implement RICM on an existing inverted bright-field or epifluorescence microscope. In particular the RICM implementation described here does not require an anti-flex objective, commonly used in other setups, which extends its versality [1]. A simplified view of the microscope is shown in Fig. S1. A white light incoherent source (Light source: HPLS345, Thorlabs) was coupled into an inverted microscope (IX71, Olympus, Japan) using a custom-built illumination arm. Briefly, the light passed through a dichroic bandpass filter (BPF) to select the appropriate wavelength range for RICM. In the experiments presented in this paper, the filter was either a single-band green filter (for combined RICM/brightfield acquisitions; filter FF01-530/43-25, Semrock) or a dual-band green/red filter (for dual color RICM acquisitions; filter FF01-534/635-25, Semrock). For the photodamage assessment experiment, the filter was remove and the full spectrum of the lamp was used (covering the range 400-750 nm). The light then passed through an aperture diaphragm limiting the illumination angle on the sample. This graduated diaphragm (SM1D12C, Thorlabs) was imaged at the entrance pupil of the objective using two achromatic doublet lenses (AC254-100-A, Thorlabs). The graduations ensured that the maximum illumination angle used for different experiments was reproducible, and was set to an illumination numerical aperture (INA) of 0.46 $[1]$.

The usual fluorescence cube in the microscope turret was replaced by polarisation optics: the light passed through a first polariser (WP25M-VIS, Thorlabs), was reflected by a polarising beamsplitter cube (PBS251, Thorlabs) and then passed through an achromatic, 15 mm-diameter imaging-flat quarter waveplate (QWP; custom-made, Fichou, France) before being focussed on the sample by an oilimmersion objective (60XO UPLSApo, Olympus, Japan). The beamsplitter cube was tilted by about 3° with respect to the optical axes to eliminate residual reflections from its faces. The axes of the quarter waveplate were positioned at 45° with respect to those of the polarizing cube, resulting in a circular polarization on the sample. Upon reflection on the sample, the circular polarization was inverted, and converted again into a linear polarisation upon a second pass through the QWP. However, this linear polarisation is orthogonal to the incoming one so that the beam passes through the polarizing beamsplitter cube towards the imaging port of the camera.

For dual-colour imaging at high speed, a home-built image-splitting setup was used to simultaneously acquire a red and a green image on the camera: a variable slit at the imaging port of the microscope (VA100/M, Thorlabs) was used to limit the field of view to half of the height of the camera sensor. The image plane at the camera port of the microscope was then imaged onto the sCMOS camera (ORCA Flash 4.0 V2, Hamamatsu) using achromatic relay lenses (ITL200, Thorlabs). In between the two lenses, the green and red images were separated using an imaging-flat dichroic mirror (FF560FDi01-25x36, Semrock) gently held through curable silicon paste (Sugru, FormFormForm, UK) to avoid any image distortion. Silver mirrors (PFSQ10-03-P01, Thorlabs) were used to orient the two images onto different regions of the camera for simultaneous imaging of red and green signals. These two images were then spectrally cleaned by dichroic filters to avoid any crosstalk between the two signals (green: FF01-531/46-25; red: FF01-629/56-25, Semrock) and recombined with another identical dichroic mirror (FF560-FDi01-25x36, Semrock). In Fig. S1, an example of combined RICM (green)/ bright-field (red) imaging is shown. For bright-field imaging, a red coloured glass filter (FGL610S, Thorlabs) was used to filter the light for the microscope illumination arm that was subsequently focussed by a long-distance 0.3 NA condenser (all Olympus, Japan). An autofocus device (CRISP system, ASI imaging, USA) was mounted on the camera port of the microscope just before the variable slit to ensure that the focus was stable throughout our experiments. Finally, a custom-built thermostated box (Digital Pixel, UK) enclosing most of the microscope was used to control the temperature at the sample.

Further details on our experimental setup and analysis regimes are available upon request. In sections S1.2, S1.7 and S1.8 we also provide details of the implementation of RICM image analysis and resulting tracking precision.

Figure S1: Schematic view of the multi-modal microscope used for imaging beads and cells.

S1.2 RICM pattern on bead as a function of bead height

RICM images are formed by the interference between light reflected from the substrate and from the polystyrene bead (Fig. S2). Multiple reflections inside the gold layer give rise to effective reflection coefficients r_1 and r_2 which are complex quantities. Around the bead centre ($r = 0$), the pattern is radially symmetric and its intensity as a function of the distance r from the centre can be written as:

$$
I(r) = A_1 e^{-\frac{r^2}{w_1^2}} + A_2 e^{-\frac{r^2}{w_2^2}} \cos(\phi_0 + \frac{2\pi n_{\text{buffer}}}{\lambda} \delta h(r))
$$
(S1)

where A_1 and A_2 account for the amplitude of the offset and of the fringes, respectively, and w_1 and w_2 for their empirical decrease with the bead distance to the surface [2]. n_{buffer} is the refractive index of the medium; the change in refractive index due to the presence of the HA film can be neglected due to its small amplitude. $\delta h(r)$ describes the geometrical path length difference between the ray reflecting on the bead and the one reflecting on the gold surface. At the point of this latter surface where the reflection occurs, and in the case of a bead of large radius (typically $> 5 \mu m$), it can be well approximated as:

$$
\delta h(r) \approx 2h(0) + \frac{r^2}{R} - \frac{3}{4} \frac{r^4}{R^3}
$$
 (S2)

where R is the bead radius. Finally, ϕ_0 accounts for the phase difference in r_1 and r_2 . Because this offset is very sensitive to the exact thickness of the gold layer, it is determined experimentally by measuring the RICM pattern obtained in the absence of the HA brush (as shown in Fig. 2).

From Equation S1, it can be seen that the RICM pattern is identical for all measured heights (h_{meas}) separated by $\frac{\lambda}{2n_{\text{buffer}}} \approx 200 \text{ nm}$ and so in order to ascertain the correct height, measurements were made at two wavelengths (534 nm, green and 635 nm, red). This method provides unambiguous determination of heights up to $\lambda_1 \lambda_2 / [2n_{\text{buffer}}(\lambda_2 - \lambda_1)] \approx 1.2 \,\mu\text{m}$ [3]. If further distances need to be measured, a third wavelength (e.g., in the 400-500nm range) could be added to extend this range to \approx 2 µm.

Figure S2: Sample structure and light reflection path. Multiple reflections occur in the gold layer, giving rise to two effective reflection coefficients, one before (r_1) and one after (r_2) reflection on the bead hovering over the functionalized surface. The value for the refractive index of the binding layer is indicative and not used in data analysis. A thin $(\sim 0.5 \text{ nm})$ titanium adhesion layer is present between the gold and the glass that is not shown in the figure, and its effects are, as for gold, incorporated in the phase offset ϕ_0 . A value of 1.334 was used for the HEPES buffer for analysis both at λ = 532 and 635 nm (i.e. dispersion was neglected).

S1.3 Additional controls for thickness determination and stability of HA brushes

Key technical aspects related to the thickness determination and stability of HA brushes were corroborated further. We found that the size of microbeads had an appreciable effect on the beadsubstrate distance (Fig. S3a), indicating that gravitational forces exerted by the beads can indent the HA brush. Importantly, this effect was negligible for bead diameters ≤ 25 µm and hence we used the 25 µm microbeads to determine HA brush thicknesses.

Secondly, validation of the illumination conditions used for image capture was performed by examination of the stability of another HA840 brush under various illumination settings. Under constant exposure of white, unfiltered light the thickness of the HA brush reduced significantly within just 1 h, demonstrating photo-damage to the surface (Fig. S3b, black bars). In contrast, under exposure of red/green filtered light (see Section S1.1), the HA brush thickness remained stable, demonstrating no photo-damage by illumination with the longer wavelengths of light (Fig. S3b, red bars).

These observations highlight that care should be taken to ensure that film thickness measurements are undertaken without film compression and that photo-damage should be assessed and limited by reducing the illumination intensity and using longer wavelengths. All subsequent measurements were thus performed with beads of diameter ≤ 25 µm imaged with either red or green light or combined red/green light.

Figure S3: (a) Compression of another (more dense) HA brush by microbeads of higher diameter ($n \ge 27$). (b) Photo-damage of another (less dense) HA brush by constant exposure to white, unfiltered light (black) and redgreen filtered light (red) ($n \ge 12$). Error bars show standard deviations.

S1.4 Estimating the elastic modulus of HA brushes from their grafting density

As discussed in detail in reference [4], contact mechanics and polymer brush theories can be combined in order to obtain the following expression for the elastic modulus of a brush:

$$
M = \frac{kT}{\xi^3 (1 - h/H_0)^2 \pi} \left[7 \left(\frac{H_0}{h} \right)^{5/4} + 5 \left(\frac{h}{H_0} \right)^{7/4} - 12 \right]
$$
(S3)

where kT is the thermal energy and h is the height of the compressed brush. Since the microbeads used here do not appreciably deform the brush (Fig. S3a), h/H_0 tends to one. In this limit of vanishing deformation, Eq. S3 tends to a finite value corresponding to the low strain elastic modulus, $M \approx 13$ $kT/(\pi \xi^3)$, as provided in the main text (Eq. 3).

S1.5 Estimating the elastic modulus of a soft film from indentation by bead gravitational force

The modulus derived from the rms distance (Eq. 3) can be compared to that obtained from data such as those provided in Fig. S3a, showing how the use of larger (hence heavier) microbeads results into compression of the HA840-h brush. As detailed in [4], the relationship between the compression of the brush and F_G , the gravitational force acting on the brush due to the bead's weight, reads:

$$
F_{\rm G} = \frac{4}{3}\pi R^3 g \Delta \rho = \frac{\pi M (H_0 - h)^2 R}{H_0}
$$
 (S4)

with $q = 9.81$ m s², $\Delta \rho$ the density difference between the bead and fluid (40 kg m⁻³ for polystyrene vs. water), and R the bead radius.

In Fig. S4, we replot the data of Fig. S3a as F_G as a function of $\pi R(H_0-h)^2/H_0$. It can be seen that the data indeed fall on a straight line, the slope of which yields the modulus M . From such an analysis, we compute $M = 35 \pm 5$ Pa, in good agreement with the value of 40 ± 18 Pa obtained from Eq. S3 and reported in the main text for the HA840-h sample.

Such a consistency check validates our use of Eq. S3 to estimate elastic moduli. It also shows that measuring the height of beads of various sizes is an easy means to probe the elastic response of a soft laver. While nominally simpler, and requiring no a priori knowledge of the molecular architecture of the layer, such a method is nonetheless limited to layers displaying moduli lower than about 1 kPa, above which the compression levels obtained with polystyrene microbeads would fall below the experimental accuracy of RICM. The use of denser (e.g., glass) microbeads would however allow probing layers of moduli up to 10 kPa.

Figure S4. Plot of the compression data from Fig. S3a as F_G vs $\pi R(H_0-h)^2/H_0$ (symbols). The red line is a linear fit to the data, yielding a slope $M = 35 \pm 5$ Pa.

S1.6 Estimating the elastic modulus of a soft film from microbead lift under flow

We here propose an alternative method to estimate the elastic modulus that also does not require any prior knowledge of the film surface density and architecture and is based on the measurement of elastohydrodynamic lift. This lift arises from the flow-induced deformation of soft films by flowing (and non-interacting) microbeads, with the magnitude of lift correlating with film softness, as demonstrated for HA brushes in Fig. S5.

A quantitative analysis of elastohydrodynamic lift on HA brushes has been described in some detail in our recent work [4], and is adapted here in order to determine the brush elastic modulus, M, from measured lift and imposed shear stress. Neglecting inertial effects, which remain small in laminar flow assays, the vertical forces acting on a flowing bead are (i) the gravitational force (F_G) and (ii) the elastohydrodynamic force (F_{EHD}) arising from the glycocalyx deformation. The balance of these two opposing forces reads:

$$
\frac{\eta^2 R^2 (V - R\Omega)^2 H_0}{M\delta^3} = \frac{4}{3} \pi R^3 g \Delta \rho
$$
 (S5)

with η the viscosity, g, $\Delta \rho$ and R as defined above, V the translational bead velocity, Ω the bead angular velocity, and δ the measured lift. In the limit where $\delta/R \ll 1$, V and Ω can in turn be expressed as a function of δ and the imposed shear rate $\dot{\gamma}$ as [4]:

$$
V = \frac{\dot{\gamma}R\left(1 - \frac{\delta}{R}\right)}{0.7625 - 0.2562\ln\left(\frac{\delta}{R}\right)} = \dot{\gamma}R \times F\left(\frac{\delta}{R}\right)
$$
(S6)

$$
R\Omega = \frac{\dot{\gamma}R}{1.6167 - 0.4474 \ln\left(\frac{\delta}{R}\right)} = \dot{\gamma}R \times G\left(\frac{\delta}{R}\right) \tag{S7}
$$

Combining Eqs. S5 to S7 yields for M:

$$
M = \frac{3\eta^2 \dot{\gamma}^2 H_0}{4\pi R^2 g \Delta \rho} f\left(\frac{\delta}{R}\right) \tag{S8}
$$

with the function $f(\delta/R) = (F-G)^2/(\delta/R)^3$, where functions F and G are defined in Eqs. S6 and S7, respectively. Furthermore, $f(\delta/R)$ can be approximated, with better than 6% accuracy for δ/R in the range from 10^{-4} to 0.3, by:

$$
f\left(\frac{\delta}{R}\right) \approx 0.11 \left(\frac{\delta}{R}\right)^{-2.827} + 1.8 \left(\frac{\delta}{R}\right)^{-1.83} \tag{S9}
$$

Substituting shear stress, $\tau = \eta \dot{\gamma}$, provides:

$$
M \approx \frac{3\tau^2 H_0}{4\pi R^2 g \Delta \rho} \left[0.11 \left(\frac{\delta}{R} \right)^{-2.827} + 1.8 \left(\frac{\delta}{R} \right)^{-1.83} \right]
$$
(S10)

Using the highest measured lift for each brush (Fig. S5), the elasticity values computed with Eq. S10 were 4 Pa (HA840-l), 40 Pa (HA840-h) and 11 kPa (HA58). These values agree rather well with the elasticities computed through Eq. 3 (Fig. 4d), validating the analysis of elastohydrodynamic lift as an alternative method to determine the elasticity of soft brushes with appreciable lift.

Figure S5. Lift (equal to the bead-wall distance after subtraction of the bead-wall distance at rest) on soft HA brushes (HAdp15 (blue), HA50 (magenta), HA840-h (green), HA840-l (orange)) measured by tracking the motion of uncoated microbeads in 3 dimensions with RICM under increasing wall shear stresses. Error bars show standard errors of the mean, which are typically smaller than the symbol size. For each shear stress ~20 beads were analysed. Data for polymer films taken from [4].

S1.7 CD44+ bead characterisation by flow cytometry

In order to estimate the density of CD44 on SuperAvidin (SuAv) beads, the biotin binding capacity of the beads was first established. SuAv coated 15 um beads were functionalised with biotinylatedfluorescein (bFITC; $M_W = 732.8$; Thermofisher Scientific, France) and analysed by flow cytometry (Fig. S6a, red dataset). The bFITC saturation curve had a plateau at an intensity at $I_{\text{bFTC}}^{\text{sat}} = 832 \pm 242$. The incubation concentration of bFITC required for saturation was 266 nM, which under the incubation conditions here (reaction volume of 50 μ L and bead concentration of 0.5% (w/v)) gives a biotin binding capacity of 40 ng of bFITC per mg of beads. This value is in good agreement with the biotin-binding capacity of 16 ng/mg reported by the supplier; slight differences are likely due to experimental conditions, in which we examined a range of concentrations rather than an exact titration. From the provider's value for the biotin binding capacity, we compute a surface density at saturation of bFITC of $\rho_{\text{bFITC}}^{\text{sat}} \approx 32000$ molecules/ μ m².

Using the bFITC data as a reference for the density of sites available on the beads provides a comparison with bCD44 coated beads. As shown in Fig. S6a, the bCD44 fluorescence level was much lower than that of bFITC. This is despite the fact that IM7-FITC used to detect bCD44 has 3-7 FITC moieties compared to bFITC possessing just 1 fluorescein moiety, according to supplier information. We hypothesize that 15 μ m beads show lower bCD44 coverage than b-fluorescein due to steric hindrance of larger bCD44 (\sim 60 kDa) binding to SuAv compared to the very small bFITC (732.8 Da). To account for the difference in FITC stoichiometry, and to relate IM7-FITC fluorescence values to surface density of CD44, we normalise the raw level of fluorescence of IM7-FITC by dividing it by a factor of 5 (the average of 3 to 7 moieties per IM7-FITC molecule), to derive comparable data with bFITC. The surface density of CD44 (ρ_{bCD4}) is then computed from the normalised IM7-FITC fluorescence intensity $(I_{\text{IM7-FITC}}^{\text{norm}})$ as:

$$
\rho_{bCD44} = \rho_{bFITC}^{sat} \frac{l_{IM7+TITC}^{norm}}{l_{bFITC}^{sat}} \tag{S11}
$$

This produced the data in Fig. S6b.

Figure S6: Characterisation of binding site density on CD44+ beads. (a) bFITC and bCD44 saturation curves of 15 µm SuAv beads. (b) Estimation of binding site density on CD44+ beads from fluorescence intensity. Error bars show rSD (normalised robust standard deviation from BD FACSDiva Software, x axis data) and calculated relative errors (y axis data). Data were measured on a LSRII flow cytometer with a FITC voltage of 400, and 10000 events per sample.

S1.8 3D tracking of beads with RICM

A schematic representation of the determination of the 3D trajectory of beads using RICM is shown in Fig. S7. First, the location of the bead centre was estimated in two dimensions using an iterative algorithm based on the symmetry of the RICM pattern: a window for pattern analysis was approximately centred around the bead, manually for the first time point of the sequence (red square in Fig. S7). This sub-image $Im1$ was then flipped with respect to its centre, yielding image $Im2$, and the correlation of two resulting images was calculated. This calculation is performed as $FT^{-1}(FT(Im1) \times FT(Im2)^*)$. where * denotes complex conjugation and TF is the Fourier Transform operation: using Fourier Transform operation ensures that the tracking can be achieved at high speed, hence allowing highthroughput analysis. The resulting cross-correlation image indicates, for all relative displacements of Im1 and Im2, how well they are superimposed. It exhibits a peak that corresponds to their best superposition displaced from its centre by a distance $[2x,2y]$ equal to twice the distance between the centre of the RICM pattern and that of the initial (red) window. x and y are then estimated with subpixel resolution by fitting the inter-correlation peak (parabolic fit on 5×5 points around the peak), which knowing the coordinates of the red window in the initial image provides the 2D coordinates of the bead in image *n*. For image $n + 1$, the window is centred around this estimated coordinate and the process is iterated, finally providing the in-plane trajectory of the bead. This method ensures that the bead can be tracked even if its RICM pattern changes due to a change in height, provided that the displacement between two images is smaller than half the size of the window. In the program the window size can be adjusted for the tracking of beads that cover a range of displacements between each frame.

For each time point, once the centre of the RICM pattern is determined, the pattern is azimuthally averaged to provide an experimental $I(r)$ curve (with r the distance from the bead centre) that is fitted using Equations S1 and S2. $h(0)$ is extracted from the difference in phase of the centre of the pattern (at $r = 0$) between the image of interest and a reference image at rest in the absence of an HA brush.

Figure S7: 3D tracking of beads in RICM images. This figure illustrates the procedure applied at each time point, which provides the in-plane position and subsequently the out-of-plane position of the bead. When iterating over the whole time sequence, one obtains the xy-trajectory with the former and the z-trajectory from the latter.

S1.9 Estimating the precision of in-plane tracking of beads

To estimate the precision of our tracking procedure in the xy plane, we recorded time sequences of noninteracting beads hovering above a SAv layer (5 beads) and a HA840-h brush (5 beads) in the absence of flow. The beads exhibit Brownian motion that can be characterized by plotting their mean square displacement (MSD), $\langle r(t+\tau) - r(t) \rangle$ (where r denotes the in-plane displacement between times t and t $+\tau$, and \lt is the average over the whole trajectory, i.e. over t), as a function of the time interval τ (Fig. S8). In the absence of flow the resulting curve should be linear with a slope $4D$, where D is the diffusion coefficient of the sphere in the medium. Here, we measured $D \approx 3.7 \times 10^{-14} \text{ m}^2 \text{ s}^{-1}$, slightly lower than the theoretical value $(5.8 \times 10^{-14} \text{ m}^2 \text{ s}^2)$ as expected for a bead hovering close to a wall. Compared to the theoretical prediction of a linear curve, however, experimental data points when excluding $\tau = 0$ exhibit a constant offset, so that the data can be fitted instead by $MSD(\tau) = \Delta r + 4 D \tau$, where Δr is the precision of the bead tracking with our algorithm. With this method, we find $\Delta r = 5.5 \pm 1$ nm rms, highlighting the good precision of our tracking procedure.

It can be noted also that the value of the diffusion coefficient can be used to estimate the fluctuations of velocity that are expected to be observed for 15 µm diameter beads tracked at 100 Hz: these fluctuations should have a standard deviation of the order of 2 to 3 μ m.s⁻¹, which corresponds to experimental observations on non-interacting beads under flow (see Fig. 6a, black dots). This confirms that the fluctuations observed on bead trajectories under flow are due to Brownian motion or interactions with the substrate rather than to the precision limit of the tracking.

Figure S8: Estimation of the tracking precision in the xy plane from RICM images. 25 s trajectories were acquired at 100 Hz (2500 images) for 10 different 15-µm-diameter beads resting on a SAv layer (5 beads) or a HA840-h brush (5 beads). Gray symbols are MSDs of individual beads while the black dots are the ensemble average over the 10 beads. The red line is a linear fit excluding the data point at time interval $\tau = 0$, and does not pass through point (0,0) due to the precision error, which can be graphically evaluated as square root of the distance at $\tau = 0$ between the red line and the black dot.

S2. Supplementary References

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