Supplementary Methods

Optical setup. SAIM imaging was performed on one of two Nikon TI-E microscopes equipped with a Nikon 60x Plan Apo VC 1.20 NA water immersion objective, and four laser lines (405, 488, 561, 640 nm), either a Hamamatsu Flash 4.0 or Andor iXon EM-CCD camera, and μManager software [5]. A polarizing filter was placed in the excitation laser path to polarize the light perpendicular to the plane of incidence. Angle of illumination was controlled with either a standard Nikon TIRF motorized positioner or a mirror moved by a motorized actuator (Newport, CMA-25CCCL).

Preparation of reflective substrates with adsorbed nanobeads. N-type silicon wafers with 1900 nm ± 5% thermal oxide were purchased from Addison Engineering. Wafers were cut to approximately 1 cm^2 using a diamond tipped pen and cleaned using air plasma for five min at a radio frequency of 18W (Harrick Plasma). 40- or 100-nm carboxylate-modified yellow-green, orange, or red fluorescent spheres (Invitrogen) were diluted in 70% ethanol, added to the wafers, and dried in a vacuum desiccator. The wafers were then washed vigorously with water, air-dried, and stored at room temperature.

Electron microscopy. 40- or 100-nm carboxylate-modified yellow-green, orange, or red fluorescent spheres (Invitrogen) were prepared by 100- to 500-fold dilution into 70% ethanol followed by sonication. To prepare grids for negative stain EM, samples were applied to freshly glow discharged carbon coated 400 mesh copper grids and blotted off. Immediately after blotting, a 2% uranyl formate solution was applied for staining and blotted off. The stain was applied five times per sample. Samples were allowed to air dry before imaging. Data were collected on a Tecnai T12 microscope (FEI) equipped with a 4K x 4K CCD camera (UltraScan 4000, Gatan). 100 nm nanobeads were imaged with a pixel size of 0.58 pixels per 1 nm and magnification of 6,500x, and 40 nm nanobeads were imaged with a pixel size of 0.98 pixels per 1 nm magnification of 11,000x.

Preparation of reflective substrates with supported lipid bilayers. Silicon wafers with 1900 nm oxide spacers were obtained from Addison Engineering, cut, and cleaned using the same process as for for nanobead imaging. Synthetic 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (POPC; Avanti, 850457), 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt, DGS-NTA-Ni; Avanti, 790404) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (ammonium salt, PEG5000-PE; Avanti, 880220) were purchased from Avanti Polar Lipids. Small unilamellar vesicles (SUVs) were prepared from a mixture of 95.5% POPC, 2% DGS-NGA-Ni, and 0.5% PEG5000-PE. The lipid mixture in chloroform was evaporated under argon and further dried under vacuum. The mixture was then rehydrated with phosphate buffered saline pH 7.4 and cycled between -80°C and 37°C 20 times, and then centrifuged for 45 min at 35,000 RCF. SUVs made by this method were stored at 4°C and used within two weeks of formation. To make labeled supported lipid bilayers, wafers were submerged in PBS in freshly plasma cleaned custom PDMS chambers on RCA cleaned glass coverslips. 100 μl of SUV solution containing 0.5 to 1 mg/ml lipid was added to the coverslips and incubated for 30 min. Unadsorbed vesicles were removed by washing three times with PBS, then bilayers were stained for 20 min with approximately 100 ng/mL DiO, DiI and/or DiD solution in PBS (Invitrogen). Wafers were again washed three times with PBS. The wafer is subsequently inverted in the imaging chamber prior to imaging.

Preparation of microtubules. Tubulin was purified from pig brain, and biotinylated or fluorescently labeled tubulin were prepared as described [Supplementary reference 11]. A mixture of unlabeled tubulin, biotin-tubulin, and fluorescent tubulin (~10:1:1 ratio) was assembled in BRB80 (80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$) + 1 mM GTP for 15 min at 37°C and polymerized MTs were stabilized with 20 μM taxol (Sigma, T1912). MTs were centrifuged over a 25% sucrose cushion to remove aggregates and unassembled tubulin before use.

Assembly of microtubule-axoneme crossings on reflective substrates. Purified, fluorescently labeled sea urchin axonemes [Supplementary reference 12] were flowed onto silicon wafers and allowed to adhere for 10 min. After washing excess unbound axonemes using BRB80 buffer (80 mM Pipes pH 6.8, 2 mM $MgCl_2$, 1 mM EGTA), the chip was coated twice with 5 mg/ml BSA-biotin (Thermo Scientific, 29130), washed with BRB80, coated with 0.5 mg/ml streptavidin (Vector Labs, SA-5000), and washed again with BRB80 plus 10 mM taxol. Polymerized microtubules were then added to the wafer and allowed to adhere for 5-10 min. Unbound microtubules were washed away using BRB80/taxol, and the wafer was then submerged in BRB80/10 μM taxol with an oxygen scavenging system [Supplementary reference 13] and inverted for imaging.

Assembly of membrane interfaces on reflective substrates. Assembly of membrane interfaces was performed according to published protocols [4], with the exception that 10x-his tagged FRB and 10x-his tagged FKBP were used in place of GFPuv. Expression and purification of recombinant human FKBP12 [Supplementary reference 14] and the FRB domain of human FRAP [Supplementary reference 15] have been described. Giant unilamellar vesicles were allowed to interact with the bilayer for 30 min to 1 hr before inverting wafer for imaging.

Modeling SAIM data in Chimera software. SAIM models were generated using Chimera software [Supplementary reference 16]. For microtubule model, SAIM data was opened in chimera as a list of x,y,z-coordinates for each pixel and a microtubule was modeled as a cylinder with radius 25 along a spline fit through the coordinates. For membrane models, SAIM topography maps (.tif) were opened as planes and the “topography” command was used to create a surface.

Supplementary References

[1] Paszek, M. J., DuFort, C. C., Rubashkin, M. G., Davidson, M. W., Thorn, K. S., Liphardt, J. T., & Weaver, V. M. (2012). Scanning angle interference microscopy reveals cell dynamics at the nanoscale. *Nature Methods*, *9*(8), 825–827. doi:10.1038/nmeth.2077

[2] Braun, D., & Fromherz, P. (1997). Fluorescence interference-contrast microscopy of cell adhesion on oxidized silicon. *Applied Physics A: Materials Science & Processing*, *65*, 341–348. doi:10.1007/s003390050589

[3] Lambacher, A., & Fromherz, P. (2002). Luminescence of dye molecules on oxidized silicon and fluorescence interference contrast microscopy of biomembranes. *Journal of the Optical Society of America B: Optical Physics*, *19*(6), 1435–1453. doi:10.1364/JOSAB.19.001435

[4] Ajo-Franklin, C. M., Ganesan, P. V, & Boxer, S. G. (2005). Variable incidence angle fluorescence interference contrast microscopy for z-imaging single objects. *Biophysical Journal*, *89*(4), 2759–69. doi:10.1529/biophysj.105.066738

[5] Chung, M., Koo, B. J., & Boxer, S. G. (2013). Formation and analysis of topographical domains between lipid membranes tethered by DNA hybrids of different lengths. *Faraday Discuss.*, *161*, 333–345. doi:10.1039/C2FD20108A

[6] Paszek, M. J., DuFort, C. C., Rossier, O., Bainer, R., Mouw, J. K., Godula, K., … Weaver, V. M. (2014). The cancer glycocalyx mechanically primes integrin-mediated growth and survival. *Nature*, *511*(7509), 319–25. doi:10.1038/nature13535

[7] Stehbens, S. J., Paszek, M., Pemble, H., Ettinger, A., Gierke, S., & Wittmann, T. (2014). CLASPs link focal-adhesion-associated microtubule capture to localized exocytosis and adhesion site turnover. *Nature Cell Biology*, *16*(6), 561–73. doi:10.1038/ncb2975

[8] Liu, J., Wang, Y., Goh, W. I., Goh, H., Baird, M. A., Ruehland, S., … Kanchanawong, P. (2015). Talin determines the nanoscale architecture of focal adhesions. *Proceedings of the National Academy of Sciences*, *112*(35), E4864–E4873. doi:10.1073/pnas.1512025112

[9] Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), 671–5. doi:10.1038/nmeth.2089

[10] Kučerka, N., Nieh, M.-P., & Katsaras, J. (2011). Fluid phase lipid areas and bilayer thicknesses of commonly used phosphatidylcholines as a function of temperature. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1808*(11), 2761–2771. doi:10.1016/j.bbamem.2011.07.022

[11] Castoldi, M., & Popov, A. V. (2003). Purification of brain tubulin through two cycles of polymerization–depolymerization in a high-molarity buffer. *Protein Expression and Purification*, 32(1), 83–88. doi:10.1016/S1046-5928(03)00218-3

[12] Gibbons, I. R., & Fronk, E. (1979). A latent adenosine triphosphatase form of dynein 1 from sea urchin sperm flagella. *The Journal of Biological Chemistry*, *254*(1), 187–96. pmid:214440

[13] Aitken, C.E., R.A. Marshall, & J.D. Puglisi. (2008). An oxygen scavenging system for improvement of dye stability in single-molecule fluorescence experiments. *Biophys J*. 94:1826-1835. doi: 10.1529/biophysj.107.117689

[14] Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L., & Clardy, J. (1993). Atomic Structures of the Human Immunophilin FKBP-12 Complexes with FK506 and Rapamycin. *Journal of Molecular Biology*, *229*(1), 105–124. http://doi.org/10.1006/jmbi.1993.1012

[15] Chen, J., Zheng, X. F., Brown, E. J., & Schreiber, S. L. (1995). Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(11), 4947–51. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7539137

[16] Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., & Ferrin, T. E. (2004). UCSF Chimera—A visualization system for exploratory research and analysis. *Journal of Computational Chemistry*, *25*(13), 1605–1612. doi:10.1002/jcc.20084