Vascular endothelial growth factor receptor 2 (VEGFR2) is a 151-kDa member of the receptor tyrosine kinase (RTK) family (1–3). This receptor is expressed on the surface of endothelial cells and controls angiogenesis, the formation of new blood vessels from existing vasculature, as well as vasculogenesis, the de novo formation of new blood vessels in tissues (4–6). Thus, VEGFR2 plays a critical role in human development and in cancer progression and is a valuable drug target. Indeed, therapies that inhibit VEGFR2 and angiogenesis would be applicable to many solid tumors, which need oxygen to grow, whereas proangiogenic therapies would be beneficial in the treatment of ischemia, such as in coronary artery disease, stroke, and chronic wounds (7–9).

VEGFR2, like most RTKs, consists of an extracellular (EC) domain, a single-pass α-helical transmembrane (TM) domain, and an intracellular kinase domain (10). VEGFR2’s EC domain is one of the largest of the RTK family and is composed of seven Ig-like domains, known as subunits D1–D7. Subunits D2 and D3 serve as the binding sites for activating ligands. As in the case of most RTKs, VEGFR2 dimerization and ligand binding are required for its activation (11–13). Dimeric, ligand-bound receptors efficiently cross-phosphorylate each other on specific intracellular tyrosines, which serve as docking sites for intracellular adaptor proteins (1, 2, 11, 14). Adaptor protein binding initiates cytoplasmic signaling cascades controlling endothelial cell survival, proliferation, and motility (1).

The ligands that bind VEGFR2 (VEGF-A and processed forms of VEGF-C and VEGF-D) are released by cells under hypoxic conditions and direct angiogenesis (2, 4, 15). Of these ligands, VEGF-A exhibits the highest binding affinity for VEGFR2 and is considered the most potent angiogenic agent. VEGF-A is a disulfide-linked, antiparallel homodimer (16) that exists as four different isoforms (121, 165, 189, and 206 amino acids long). Although the binding strengths of all VEGF-A isoforms to VEGFR2 are thought to be the same, these isoforms differ in their interactions with the extracellular matrix and the coreceptors on the surface of cells (14, 17–20). VEGF-A121 is the smallest isoform of VEGF-A; however, it contains the full VEGFR2-binding site but lacks the sites mediating the interaction with the extracellular matrix and other coreceptors.

It has been found that VEGFR2 can exist in either a monomeric or a dimeric form on the plasma membrane of cells, even in the absence of ligand (21–23). The existence of VEGFR2 monomers and dimers on the plasma membrane suggests that the formation of VEGF-bound, active dimers can occur through several different pathways (see Fig. 1). In one pathway, the monomeric receptors dimerize on the plasma membrane, upon which a ligand may then bind to the preformed dimer and activate it. Alternatively, a ligand may bind to a monomer of VEGFR2. This liganded monomer may then dimerize with an unliganded VEGFR2 monomer to form an active liganded VEGFR2 dimer. At very high VEGF concentrations, a third pathway exists in which two ligand-bound VEGFR2 monomers can dimerize to form an active dimer upon release of one of the bound VEGF ligands. The prevalence of these pathways in an experiment designed to measure VEGF–VEGFR2 binding affinities will depend on the VEGF2 ligand-free monomer–
dimer association constant and thus on the total concentration of VEGFR2 on the plasma membrane of cells, on the possibly different binding affinities of VEGF for monomeric and dimeric forms of VEGFR2, and on the concentration of free VEGF in the medium around the cells.

There are previous reports of measurements of effective VEGF binding constants, but they do not discriminate between the association state of the receptor on the surface of the cell because they do not account for the surface density dependence of VEGFR2 dimer formation on the plasma membrane (24–27). Thus, most methods used to assess ligand binding to cell surface receptors cannot provide an answer as to which of the pathways is preferentially utilized in the VEGF–VEGFR2 system. Here, we overcome these prior methodological limitations by employing the fully quantified spectral imaging (FSI) methodology (22) to directly measure the surface density of expressed VEGFR2, the surface density of bound VEGF on the same cell, and the free VEGF concentration in the buffer surrounding the cells. Imaging many cells at different free VEGF concentrations enables the use of a rigorous thermodynamics approach and global fitting to simultaneously determine the separate affinities of VEGF for monomeric and dimeric forms of VEGFR2.

To perform these measurements, we use a commercially available Alexa Fluor 594–labeled version of a recombinant single-chain derivative of vascular endothelial growth factor (scVEGF) (28). Although VEGF-A is a disulfide-linked dimer of two separate chains, scVEGF is an engineered fusion protein that combines two fragments, each composed of amino acids 3–112 of VEGF-A121, that are cloned consecutively in a head-to-tail fashion. By creating a single recombinant protein, Backer and Backer (29) were able to introduce an N-terminal 15-amino-acid tag containing a unique cysteine residue that could be used for site-specific attachment of a single fluorophore despite the presence of 16 native cysteines. scVEGF is a fully functional form of VEGF-A121 as it binds and activates VEGFR2 just like endogenous VEGFs. It is widely used in translational research and has already proven its utility as an imaging and a therapeutic agent (28, 30, 31).

Results

Theory: A thermodynamic cycle for VEGF binding to VEGFR2

We utilize a thermodynamic cycle that accounts for all of the different forms of VEGFR2 that can exist in the absence and presence of VEGF: monomers (M), dimers (D), ligand-bound monomers (LM), and ligand-bound dimers (LD) as shown in Fig. 1A. This model assumes that one molecule of VEGF can bind either a VEGFR2 monomer or a VEGFR2 dimer. This thermodynamic cycle includes all the possible pathways leading from unliganded VEGFR2 monomers to active, VEGF-bound VEGFR2 dimers. Among the top left of the cycle, VEGF can undergo unliganded dimerization with affinity $K_L$ in units of (receptors/µm²)⁻¹.

\[
K_L = \frac{[D]}{[M]^2}
\]  (Eq. 1)

VEGF can then bind to the preformed dimer of VEGFR2 with affinity $K_{LM}$, having inverse molar units (M⁻¹).

Alternatively, a VEGF ligand in solution can bind to a monomer of VEGFR2 with affinity $K_{LM}$ (M⁻¹) (moving from the top left position in the thermodynamic cycle down in a counterclockwise direction in Fig. 1A).

\[
K_{LM} = \frac{[LM]}{[L][M]}
\]  (Eq. 3)

Moving right in the cycle, a VEGF-bound VEGFR2 monomer, LM, can dimerize with an unliganded VEGFR2 monomer, M, again forming the ligand-bound, active dimeric form of VEGFR2 with an affinity of $K_{LMD}$, having units of (receptors/µm²)⁻².

\[
K_{LMD} = \frac{[LMD]}{[LM][M]}
\]  (Eq. 4)

Traversing further counterclockwise around the cycle, under saturating VEGF conditions two liganded VEGFR2 monomers, (LM), can combine to form a liganded dimer of VEGFR2 (LD) only upon release of a VEGF molecule into solution. This process is described by the association constant $K_{LM–LD}$ with units of M²(receptors/µm²)⁻¹.

\[
K_{LM–LD} = \frac{[LM–LD]}{[LM][M]}
\]  (Eq. 2)
Equation 9 provides a link between the measured cells at different $[L]_{\text{free}}$ concentrations yield an overdetermined system of equations that is used to determine the best-fit values of the monomeric and dimeric binding affinities, $K_{L,M}$ and $K_{L,D}$.

$K_{L,M}$ and $K_{L,D}$ are measured previously (21, 22), and the measured value is given in Table 1.

**Experimental measurements of molecular binding affinities**

We seek to measure the two-dimensional VEGFR2 surface density in the plasma membranes of HEK293T cells as well as the concentration (surface density) of the bound scVEGF. To achieve this goal, we subject the cells to reversible osmotic swelling as described previously (22, 32, 33). This treatment is required for quantitative determination of the concentration of receptors and bound ligands on the surface of the cells as the complex topology of the resting plasma membrane prevents the conversion of effective 3D concentrations into 2D membrane protein surface densities (22). The osmotic swelling is completely reversible (34–36) and does not change the molecular interactions of VEGFR2 ECTM constructs in the plasma membrane (22). We use a VEGFR2 construct, VEGFR2 ECTM-YFP, in which the kinase domain is substituted with YFP attached via a (GGS)₅ flexible linker to the TM domain (see Discussion below).

Fluorescent AF594-scVEGF is added to the hypotonic swelling medium, and cells expressing VEGFR2 are imaged under two-photon excitation with the OptiMis spectral detection system (37, 38). As shown in Fig. 2A, YFP is intracellularly located, whereas AF594-scVEGF is bound to the extracellular domain of VEGFR2. Fig. 2B shows a schematic of the experimental setup. Measurements of the plasma membrane VEGFR2 surface densities, bound VEGF surface densities, and free VEGF concentrations in the media are performed with the FSI methodology. As described previously (22), FSI enables the measurements of the surface density of fluorophore-labeled membrane proteins in 2–3-μm-size patches by performing two scans, a “donor scan” to excite YFP at 960 nm and an “acceptor scan” to excite AF594 at 800 nm. The fluorescence absorbance and emission spectra of YFP and AF594 are shown in Fig. 2C. FSI also allows for the measurement of the three-dimensional concentration of freely diffusing, fluorescent moieties, in this case free AF594-scVEGF, in the solution surrounding the cells.

**Table 1**

Thermodynamic parameters describing VEGF binding to VEGFR2

| Association constant                          | ECTM VEGFR2                  | Full-length VEGFR2               |
|-----------------------------------------------|------------------------------|----------------------------------|
| $K_{L,M}$ ($M^{-1}$)                         | $9.6 \times 10^8 \pm 1.8 \times 10^7$ | $9.6 \times 10^8 \pm 1.8 \times 10^7$ |
| $K_{L,D}$ ($M^{-1}$)                         | $4.3 \times 10^9 \pm 0.6 \times 10^9$ | $4.3 \times 10^9 \pm 0.6 \times 10^9$ |
| $K_{LM}$ ($rec/\mu m^2$)$^{-1}$              | $3.7 \times 10^{-6} \pm 0.6 \times 10^{-6}$ | $3.7 \times 10^{-6} \pm 0.6 \times 10^{-6}$ |
| $K_{LM} = K_{L,M}K_{L,D}/K_{LM}$ ($rec/\mu m^2$)$^{-1}$ | $1.7 \times 10^{-2} \pm 3.9 \times 10^{-3}$ | $1.3 \pm 0.3$ |
| $K_{LM,D} = K_{L,M}K_{L,D}/K_{LM}$ ($rec/\mu m^2$)$^{-1}$ | $1.7 \times 10^{-10} \pm 1.3 \times 10^{-8}$ | $1.4 \times 10^{-7} \pm 1.2 \times 10^{-6}$ |

* Measured previously (21, 22).
measured total apparent FRET efficiency between the intracellular YFP of VEGFR2 ECTM and the extracellularly bound AF594-scVEGF as a function of total VEGFR2 surface density. The FRET efficiency is zero for all concentrations of VEGFR2. This is expected because YFP (the FRET donor) and the AF594 (the FRET acceptor) are further than 10 nm apart when VEGF is bound to the distal region of VEGFR2’s EC domain (see Fig. 2A). Indeed, the plasma membrane is ~5 nm thick on its own, and the intracellular YFP is a large β-barrel connected to VEGFR2 TM domains via a 15-amino-acid (GGS)5 flexible linker, whereas VEGF is bound to the extracellular D2 and D3 subunits of VEGFR2. Thus, the negligible FRET efficiencies measured for all VEGFR2 surface densities and all free VEGF concentrations serve as an important control for these experiments.

Fig. 5 shows the bound AF594-VEGF surface density plotted as a function of the expressed VEGFR2 ECTM-YFP surface density for each of the 12 different free VEGF concentrations. There is a clear dependence of the binding of VEGF to VEGFR2 on the three-dimensional free scVEGF concentration, where the addition of more free AF594-scVEGF to the media results in more VEGF binding to the VEGFR2 ECTM-YFP construct.

We combined the data from the 12 data sets in Fig. 5 and performed a global fit using Equations 8 and 9 to find the best-fit values of the two adjustable parameters, the binding affinity of AF594-scVEGF for monomeric VEGFR2, $K_{LM}$, and the binding affinity of AF594-VEGF for dimeric VEGFR2, $K_{LD}$ (see also Fig. 1). These are the best-fit values of $K_{LM}$ and $K_{LD}$ that globally minimize the error of the model for all 12 data sets, significantly enhancing the accuracy and precision of the fit over that obtained by fitting experiments individually. We find that $K_{LM} = 9.6 \pm 1.8 \times 10^{-7}$ M$^{-1}$ and that $K_{LD} = 4.3 \pm 0.6 \times 10^{-9}$ M$^{-1}$ (95% confidence). Thus, there is a 45-fold enhancement of VEGF binding affinity for dimeric VEGFR2 over the affinity of AF594-VEGF for monomeric VEGFR2. The corresponding dissociation constants are 10 nm for VEGF binding to monomeric VEGFR2 and 230 pm for binding of VEGF to dimeric VEGFR2.

The dependence of the bound VEGF as a function of expressed VEGFR2 ECTM surface density for each free VEGF concentration is plotted in the red solid lines of Fig. 5. The dashed red lines indicate the confidence limits on the fit, obtained through propagation of errors on $K_{LM}$ and $K_{LD}$ in Equations 8 and 9, and the 95% confidence limits on $K_{LM}$ and $K_{LD}$. We see good agreement between the measured data and the best-fit predicted bound VEGF surface densities as a function of VEGFR2 surface densities over 2 orders of magnitude of free VEGF concentrations. Thus, the thermodynamic cycle in Fig. 1 is a model that explains our measured data over a wide range of experimental conditions.

Now that $K_{LM}$ and $K_{LD}$ are determined, we can calculate the dimerization constants $K_{LMD}$ and $K_{LM-LD}$, provided that the unliganded dimerization constant $K_{R}$ is measured. We have
previously measured $K_R$ for both the ECTM VEGFR2 and the full-length VEGFR2 in the plasma membrane (21, 22), and we use the measured values for the calculations. The results are shown in Table 1. To calculate $K_{LMD}$ and $K_{LM–LD}$ for the full-length VEGFR2 receptor, we make the assumption that the measured ligand binding constants $K_{LM}$ and $K_{LD}$ are not significantly affected by the presence of the kinase domain positioned on the opposite part of the membrane. Such an assumption is justified as the binding site on the receptor is restricted to subdomains D2 and D3, far from the plasma membrane. Note that although these ligand binding constants are assumed to be independent of the kinase domains, the total bound ligand is enhanced by the presence of the kinase domain as the contacts between the kinase domains in the VEGFR2 dimer are stabilizing and promote dimerization. Effectively, the presence of the kinase domain enhances VEGF binding because ligand binding and dimerization are coupled.

Because $K_R$ (for unliganded VEGFR2 dimerization) and $K_{LMD}$ (for dimerization of an unliganded monomer with a ligand-bound monomer) have the same units in Table 1, their magnitudes can be directly compared. $K_{LMD}$ is 45 times greater than $K_R$, indicating that at low VEGF concentrations, dimerization is driven by VEGF binding to VEGFR2 monomers.

Given all the values in Table 1, we can now calculate the probabilities of the full-length VEGFR2 receptor to exist in different dimeric states in the plasma membrane. In particular, Equations 8 and 9 and the previously measured association constant $K_R$ for full-length VEGFR2 dimerization, $3.0 \times 10^{-2} \text{ (rec/µm}^2\text{)}^{-1}$ (21), can be utilized to predict the abundance of monomers, dimers, VEGF-bound monomers, and VEGF-bound dimers of VEGFR2 for any surface density of the full-length receptor and for any free VEGF concentration.

Figure 6, A–D, shows the predicted fractions of monomeric VEGFR2, $[M]/[T]$; VEGF-bound monomeric VEGFR2, $[LM]/[T]$; dimeric VEGFR2, $[2D]/[T]$; and VEGF-bound dimeric VEGFR2, $[2LD]/[T]$, as functions of total VEGFR2 surface density and free VEGF concentration. These fractions vary from 0 to 1 and sum up to 1 in all cases. In Fig. 7, we show these distributions at two constant receptor concentrations, 10 and 100 receptors/µm$^2$.

An important prediction of the model is the bell-shaped dependence of the active, ligand-bound, dimeric fraction of VEGFR2 on the free VEGF concentration, shown in Fig. 6D. For fixed VEGFR2 surface densities, the ligand-bound dimeric VEGFR2 fraction increases with increasing VEGF concentration at low VEGF concentrations (up to about 10 nM). At higher concentrations of VEGF, the fraction of ligand-bound dimers decreases, whereas the ligand-bound VEGFR2 monomers become dominant. Thus, there exists a ligand concentration for which VEGFR2 activity is the highest. This optimal VEGF concentration is roughly equal to the ligand–monomer dissociation constants, 10 nM, over a broad range of receptor concentrations.

Discussion

The goal of this work was to measure the binding affinity of VEGF for VEGFR2 monomers and VEGFR2 dimers and to characterize the effect of VEGF binding on VEGFR2 dimer stability. We achieve this through direct measurements of the bound VEGF surface density, the VEGFR2 surface density, and the free VEGF concentrations in 12 independent experiments. We then utilized global analysis to fit all the data to the complete thermodynamic cycle in Fig. 1.

The thermodynamic cycle depicted in Fig. 1 describes both receptor–ligand and receptor–receptor interactions, which are coupled, and accounts for all the possible forms of unliganded VEGFR2 and VEGF-bound VEGFR2 that can exist on the plasma membrane of cells. We measured the dissociation constant for VEGF binding to monomeric VEGFR2, $K_{LM}$, as 10 nM and the dissociation constant for
VEGF binding to dimers of VEGFR2, $K_{D_{L}}^{D}$, as 230 pm. Previously, only effective binding constants have been measured, with values ranging from 75 to a few nm (24–27). Here, we find that VEGF binds to both monomers and dimers of VEGFR2 and that the binding of VEGF to dimeric VEGFR2 is 45 times more favorable than the binding to monomeric VEGFR2, indicating that cooperative interactions stabilize the ligand-bound dimer.

Figure 4. The results of the free VEGF measurements for each of the 12 independent experiments. A Gaussian distribution was fitted to each histogram, yielding the best-fit mean and standard deviations for each measurement.
The equilibrium constant describing dimerization in the absence of VEGF, $K_R$, has been measured previously for full-length VEGFR2 and for truncated VEGFR2 constructs lacking the cytosolic domain (21, 22), and we utilize this knowledge in our experimental design. Full-length VEGFR2 has a very strong propensity for dimerization in the absence of ligand, with a two-dimensional dissociation constant of $33 \pm 23 \text{rec/\mu m}^2$. Under the conditions of our experiments (VEGFR2 expression between 100 and 5,000 rec/\mu m$^2$), full-length VEGFR2 exists as a nearly constitutive dimeric form, making it difficult for us to measure the affinity of the ligand to the monomeric form of VEGFR2. In contrast, the VEGFR2 ECTM construct, which lacks the intracellular kinase domain, has a much higher dissociation constant and thus lower self-affinity ($2700 \pm 430 \text{rec/\mu m}^2$), and the dimeric fraction of VEGFR2 ECTM ranges from 0 to about 80% under the same conditions. The reduced dimer stability of the truncated VEGFR2 ECTM receptor is due to loss of stabilizing contacts that occur between the two intracellular domains in the full-length VEGFR2 dimer (21).

The truncated VEGFR2 ECTM-YFP version offers additional advantages as it does not undergo endocytosis upon ligand binding and is expressed over a broader concentration range than full-length VEGFR2 in transient transfection experiments. Because the contribution of the intracellular domain to unliganded receptor dimerization is known (21), the measurements of $K_{LM}$ and $K_{LD}$ allow us to predict the behavior of the full-length VEGFR2 as a function of VEGFR2 surface density and free VEGF concentrations.

The predictions shown in Fig. 5 document how VEGFR2 surface density and free VEGF concentration control the abundance of the monomers and dimers of VEGFR2 in their VEGF-bound or unbound state. Because VEGFR2 ligand-bound dimers are the signaling-competent dimers, these predictions...
can be used to predict VEGFR2 activity. Indeed, it has been shown that unliganded VEGFR2 dimers are only weakly phosphorylated (21, 40). Ligand binding initiates a conformational switch in the VEGFR2 dimer that is translated through the TM domains to the kinase domains, ultimately ensuring high phosphorylation efficiencies and efficient downstream signaling (21). We see a bell-shaped curve describing the abundance of liganded VEGFR2 dimers as a function of total VEGFR2 surface density. Thus, we predict that high VEGF concentrations suppress VEGFR2 activation. It is noteworthy that VEGFR2 in vitro biochemical studies typically utilize concentrations of about 1 nM VEGF. At 1 nM free VEGF concentration, we predict that about 40% of the receptors exist as liganded dimers, and thus measurements of effective binding constants will be slightly weighted toward the monomeric VEGFR2 binding affinity, $K_{LM}$.

In the human body, VEGF is believed to act at free VEGF concentrations in the picomolar range (42). At these concentrations, we predict a very low fraction of ligand-bound, active VEGFR2 dimers (~1%) for all surface densities of VEGFR2 (see Fig. 6 or 7). The discrepancy is well-documented in the literature and may be due to the interactions of VEGF with the 3D extracellular matrix as opposed to 2D cell culture or possibly due to the effects of accessory proteins like NRP1 that also bind VEGF on orthogonal binding sites (33, 43, 44). These additional VEGF interactions in vivo may prolong signaling, or they may simply serve to enhance the local effective concentration of VEGF or VEGFR2. Fig. 8 shows the $x$-component of the gradient of the fraction of liganded VEGFR2 dimers (Fig. 6D), a measure of the sensitivity of VEGFR2 activity to changes in free VEGF concentration. We find that the maximum in this gradient occurs at high VEGFR2 surface density where VEGFR2 is primarily dimeric ($>10^5$ receptors/µm²) (45, 46) and at very low free VEGF concentrations ($<10^{-11}$ M). It can be expected that this is the “sweet spot” for VEGF to exert its biological action, as small changes in free ligand concentration lead to the largest changes in the population of active, ligand-bound VEGFR2 dimers. Curiously, the free VEGF concentration in this sweet spot is similar to the concentration found in vivo.

**Figure 6.** Predicted ligand-free and ligand-bound VEGFR2 fractions for full-length VEGFR2 are plotted as a function of VEGFR2 surface density and free VEGF concentration. A, ligand-free monomeric VEGFR2 fraction. B, ligand-bound monomeric VEGFR2 fraction. C, ligand-free dimeric VEGFR2 fraction. D, ligand-bound dimeric VEGFR2 fraction. At all points, the four fractions sum to 1.
Thus, small changes in the \textit{in vivo} VEGF concentration will lead to the largest possible changes in the fraction of ligand-bound active VEGFR2 dimers. This is a new concept in VEGF research which merits further investigation.

It is noteworthy that the average receptor concentration in endothelial cells is known to be less than $10^2$ receptors/$\mu$m$^2$ (45, 46), seemingly very different from $10^3$ receptors/$\mu$m$^2$ predicted for this “sweet spot” of VEGF activity. It is possible, however, that VEGFR2 is not distributed homogeneously on the cell surface but instead can reach high local concentrations in membrane domains where VEGFR2 exists as a preformed dimer and is poised to become activated upon ligand binding. Still, the maximum of the gradient occurs where the fraction of ligand-bound VEGFR2 dimers is very low, only about 1%, for all VEGFR2 expression levels. This may suggest that the cellular response in the body is exceptionally sensitive to very small numbers of activated VEGFR2 receptors.

Materials and methods

We utilized scVEGF conjugated to the organic dye Alexa Fluor 594, purchased from SibTech Inc. (catalog number SBT309). The scVEGF conjugate (molecular mass, 28 kDa) consists of two fragments of human VEGF-A121 (amino acids 3–112) cloned head to tail and fused to an N-terminal Cys tag (SibTech Inc., catalog number SBT301). The AF594-scVEGF conjugate is singly labeled in a site-specific manner at the Cys tag and exhibits 95–100% VEGF activity (28). We used a VEGFR2 ECTM construct, labeled with YFP, that has been described previously (22, 23, 33).

Cell culture and transient transfection

The HEK293T cells used in the experiments here were a kind gift from Dr. D. Wirtz, Johns Hopkins University. Transient transfections were performed with a total of 3 $\mu$g of plasmid DNA, using Lipofectamine 3000 (Invitrogen) transfection reagent, according to the manufacturer’s protocol.

The cells were serum-starved for at least 12 h prior to the application of reversible osmotic stress and imaging as described (22). At the time of serum starvation, the dishes were blocked with 0.1% BSA to prevent nonspecific binding of VEGF. A 0.1% BSA concentration was maintained throughout the rest of the experiment.

Reversible osmotic swelling, addition of VEGF to cells, and imaging

The hypotonic swelling medium was composed of serum-free medium diluted 1:9 with deionized H$_2$O, buffered with 25 mM HEPES, supplemented with 0.1% BSA, and 0.2-$\mu$m sterile-filtered. Purchased AF594-scVEGF was resuspended in PBS buffer at a concentration of 1 mg/ml and stored in aliquots of $\sim$2.5 $\mu$g/aliquot at $-20$ °C. These aliquots were then used at 1:1, 1:5, 1:10, 1:15, 1:20, 1:25, and 1:50 dilutions and mixed with swelling medium. Just prior to imaging, the starvation medium
was aspirated from the Petri dishes and gently replaced with 1 ml of 37 °C hypotonic swelling medium containing AF594-scVEGF. The cells in each dish were allowed to stabilize for at least 15 min before imaging, and images were acquired at room temperature for up to 2.5 h per dish postswelling.

**Image acquisition**

The FSI method is utilized in this work to measure the bound AF594-scVEGF surface densities, the VEGFR2 ECTM-(GGS)₆-YFP surface density, and the three-dimensional concentration of free AF594-scVEGF in the buffer surrounding the cells in the dish. The FSI methodology has been described previously in detail (22).

Spectral images were acquired with a two-photon microscope comprising a Mai Tai laser (Spectra Physics), an OptiMis True Line Spectral Imaging system (Aurora Spectral Technologies), and a Zeiss Observer wide-field microscope with a 63X numerical aperture 1.2 water immersion objective as described previously (22). In this work, two different FSI experiments were performed per dish. In the first experiment, two images of each cell were acquired, a donor scan to excite YFP at 960 nm and an acceptor scan to excite AF594-scVEGF at 800 nm. In the second experiment, images of buffer fluorescence, in a region of the dish where no cells were located, were acquired for determination of the free VEGF concentrations.

**Fully quantified spectral imaging**

Below, we show the equations that are the foundation of the FSI methodology.

\[ E_{app} = \frac{F_D}{F_{RET, A1}}/\frac{F_{A1}}{F_{app}} = 1 - \frac{F_{DA}}{F_D} = 1 + \frac{F_{A2}}{F_{A2}} \left( 1 - \frac{F_{DA}}{F_D} \right) \]  \hspace{1cm} (Eq. 10)

\[ [D] = \frac{F_{A1}}{i_{0,A1}} = \frac{1}{i_{0,A1}} \left( \frac{F_{A1}}{Q} \right) \left( 1 + \frac{F_{DA}}{F_D} \right) \]  \hspace{1cm} (Eq. 11)

\[ [A] = \frac{F_{A2}}{i_{0,A2}} = \frac{1}{i_{0,A2}} \left( \frac{F_{A2}}{Q} \right) \left( 1 - \frac{F_{DA}}{F_D} \right) \]  \hspace{1cm} (Eq. 12)

In these equations, \( E_{app} \) is the measured total apparent FRET efficiency, and \( F_{RET, A1} \) is the donor fluorescence that is transferred to an acceptor upon excitation in the donor/FRET scan at \( \lambda_1 \). \( F_{A1} \), the total fluorescence of the donor or acceptor, is measured in the absence of FRET upon excitation at \( \lambda_1 \) or \( \lambda_2 \). \( F_{DA} \) is the measured fluorescence of the donor in the presence of acceptors, and \( F_{A2} \) is the measured fluorescence of the acceptor, enhanced due to FRET. \( i_{0,A1} \) and \( i_{0,A2} \) are the slopes of the solution standard intensity versus micromolar concentration curves determined by imaging solution standards of known concentration, described below. \( Q_D \) and \( Q_A \) are the quantum yields of the donor and the acceptor.

**Equation 12** is directly applied to buffer fluorescence images, and the measured pixel-level concentrations are histogrammed. A Gaussian curve is fit to the data to determine the value of the mean, \( \mu \), and the standard deviation, \( \sigma \), yielding the mean value and error estimate of the free ligand measurement.

For the surface density measurements, the apparent pixel-level fluorophore (receptor and bound ligand) concentrations calculated during the image analysis are integrated across diffraction-limited membrane segments to determine the 2D surface density from the fluorescence and the calibration curves. To do so, \( P_D \), \( P_A \), and \( P_{DA} \) are integrated (summed) over every pixel selected in the region, \( F_{DA} \), and the apparent FRET efficiency of the region is then calculated as

\[ E_{app} = 1 - \frac{F_{DA}}{F_D} \]

The total integrated fluorescence intensities for the region, \( F_D \) and \( F_A \), are divided by the arc length, \( s \), of the selected region to calculate the average integrated fluorescence per unit length of membrane (in units of pixels). By swelling the cells with osmotic stress, we are able to simplify the complex topology of the membrane and ensure a perpendicular orientation of the membrane with respect to the focal plane. We also assume that the fluorescence originates from an infinitely thin sheet within the width of one pixel, or 254 nm. To obtain the fluorescence that is emitted by fluorophores in a voxel, the integrated fluorescence per unit pixel length is multiplied by the pixel width, 254 nm. By dividing the voxel fluorescence by the average slope, \( i_{0,A1} \), and performing the appropriate unit conversion from micromolar concentrations to receptors per unit area (in units of rec/nm²), the average receptor surface density for the region is calculated as shown below.

\[ [D or A] = \frac{[rec]}{[nm²]} = \sum_{s<}^{<} \frac{F_{DA}}{F_{DA}} \left( \frac{counts}{pixel} \right) \left( \frac{counts}{µM} \right)^{-1} \left( \frac{rec}{nm²} \right) \cdot 254 \left( \frac{nm}{pixel} \right) \]

**YFP and Alexa Fluor 594 solution standards**

Soluble monomeric YFP with an N-terminal His₆ tag was expressed and purified to near-millimolar concentrations as described (47). Fluorescent protein stocks were buffer-exchanged into PBS buffer with a 20-kDa-molecular-mass-cutoff concentrator (Pierce, catalog number 87751) and filtered with a 0.2-µm syringe filter. Unconjugated Alexa Fluor 594 (A37572) was purchased in lyophilized form from Thermo Fisher Scientific and suspended in PBS buffer. For each imaging session, the stocks were diluted in buffer to micromolar concentrations to produce 100, 75, and 50% fluorescent protein and AF594 solution standards. The solution standard concentrations were measured in a 1-cm-path-length quartz cuvette using NanoDrop 2000C (Thermo Scientific). Molar absorption coefficients of 83,400 and 73,000 M × cm⁻¹ were used to calculate the concentrations of the solution standards from the YFP and AF594 absorption maxima of 514 and 590 nm, respectively. Images of the 100, 75, and 50% solution standards and a PBS buffer-only control were acquired at both excitation wavelengths (800 nm for AF594 and 960 nm for YFP) and used for the calculation of the pixel-level slope values (22). Fig. 2C shows the normalized YFP and AF594-VEGF fluorescence obtained from the membranes of live cells. We see no apparent change in the fluorescence emission properties in the AF594-VEGF or YFP fluorophores on the surface of the cells when compared with that of the solution standards, and the respective emission maxima of 618 and 527 nm remain unchanged.
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