A monovalent streptavidin with a single femtomolar biotin binding site

Mark Howarth[1], Daniel J-F Chinnapen[1,4], Kimberly Gerrow[2,4], Pieter C Dorrestein[3], Melanie R Grandy[1], Neil L Kelleher[3], Alaa El-Husseini[2] & Alice Y Ting[1]

Streptavidin and avidin are used ubiquitously because of the remarkable affinity of their biotin binding, but they are tetramers, which disrupts many of their applications. Making either protein monomeric reduces affinity by at least 10^4-fold because part of the binding site comes from a neighboring subunit. Here we engineered a streptavidin tetramer with only one functional biotin binding subunit that retained the affinity, off rate and thermostability of wild-type streptavidin. In denaturant, we mixed a streptavidin variant containing three mutations that block biotin binding with wild-type streptavidin in a 3:1 ratio. Then we generated monovalent streptavidin by refolding and nickel-affinity purification. Similarly, we purified defined tetramers with two or three biotin binding subunits. Labeling of site-specifically biotinylated neuroligin-1 with monovalent streptavidin allowed stable neuroligin-1 tracking without cross-linking, whereas wild-type streptavidin aggregated neuroligin-1 and disrupted presynaptic contacts. Monovalent streptavidin should find general application in biomolecule labeling, single-particle tracking and nanotechnology.

Streptavidin and avidin bind the small molecule biotin with femtomolar affinity[1]. This tight and specific binding has caused the (strept)avidin-biotin system to be used widely for biomolecule labeling, purification, immobilization and patterning. But because streptavidin and avidin are both tetramers, they can tetramerize the biotin conjugates to which they bind. This multimerization can interfere with normal biomolecule function or trafficking, or complicate affinity measurements, limiting the applications of this system. Much effort has been made to develop monomeric (strept)avidin. Disruption of the tetramer interface is straightforward, but is always accompanied by a dramatic decrease in biotin affinity (at least 10^4-fold in the best case for streptavidin[2] and 10^8-fold for avidin[3,4]) because the biotin binding site lies at the interface between subunits[5,6]. An alternative approach to generate monovalent (strept)avidin is to titrate in three equivalents of biotin per tetramer, but this is unsatisfactory because it generates a statistical mixture of mono-, di-, tri- and tetravalent (strept)avidins (see **Supplementary Methods** online).

We recently reported the use of site-specific protein biotinylation and streptavidin labeling to image cellular proteins[7]. This method can be used to specifically tag proteins at the cell surface, requires genetic fusion of the protein of interest to only a 15-amino-acid peptide, provides a stable linkage that does not dissociate over hours and can be used for introduction of a wide range of probes, from organic fluorophores to quantum dots. We were concerned, however, that the streptavidin could cross-link biotinylated cell-surface proteins (such as the AMPA receptor used in our earlier study[7]), thus slowing their trafficking and causing unwanted receptor activation[8]. Here we report the development of a monovalent streptavidin with a single femtomolar biotin binding site, and its application to cross-linking–free labeling of biotinylated neuroligin-1 on the surface of living neurons.

RESULTS

Generation of monovalent streptavidin

Our strategy for producing monovalent streptavidin is shown in **Figure 1a**. We wanted to produce a streptavidin tetramer consisting of three subunits unable to bind biotin and one subunit that binds biotin as well as wild-type streptavidin. Many of the known mutations in streptavidin reduce biotin affinity dramatically[2,6,9] but still leave dissociation constant (K_d) values in the nanomolar range and disrupt tetramerization[2]. The double mutant N23A, S27D has one of the weakest reported affinities for biotin ($K_d = 7 \times 10^{-5}$ M)[10] while still remaining a tetramer. Nevertheless we observed that N23A, S27D streptavidin still bound to biotinylated cells (data not shown). We found that mutation of an additional amino acid in the biotin binding site yielded a triple mutant (N23A, S27D, S45A; **Fig. 1b**) with negligible biotin binding, but left the tetramer structure intact. The biotin affinity of this mutant (composed of 'dead' (D) subunits; **Fig. 1**) was so weak that it was difficult to measure, but we obtained an approximate K_d of 1.2×10^{-3} M. To generate monovalent streptavidin (**Fig. 1c**), we added a 6His tag to the wild-type subunit ('alive' (A) subunit; **Fig. 1**), then combined D and A subunits at a molar ratio of 3:1 in guanidinium hydrochloride and refolded them by rapidly diluting the mixture into phosphate buffered saline (PBS). This refold

[1]Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA. [2]Department of Psychiatry, the Brain Research Center, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada. [3]Department of Chemistry, University of Illinois Urbana-Champaign, Urbana, Illinois 61801, USA. [4]These authors contributed equally to this work. Correspondence should be addressed to A.Y.T. (ating@mit.edu).

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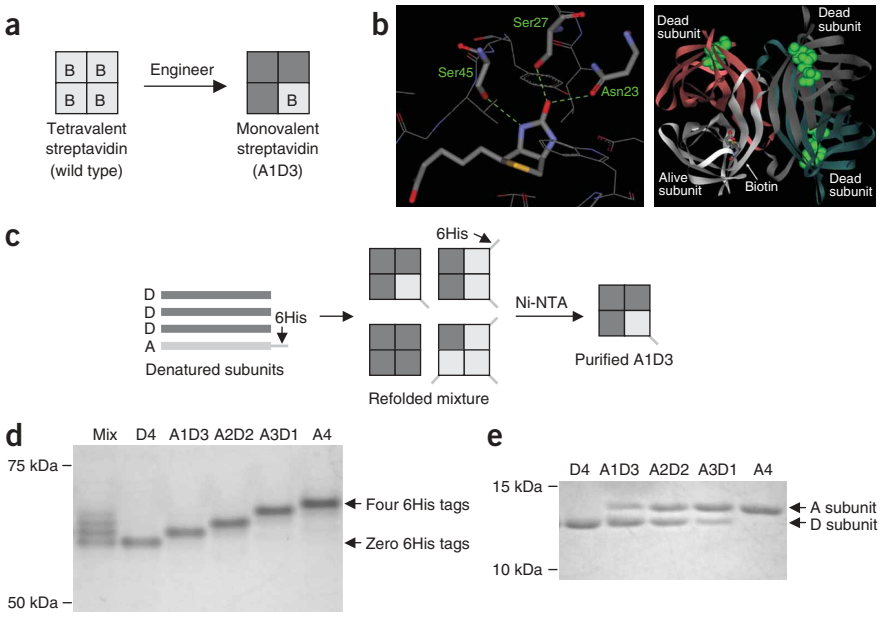

Figure 1 | Generation of monovalent streptavidin. (**a**) Wild-type streptavidin is a tetramer with four biotin binding sites (B, biotin). Monovalent streptavidin is a tetramer with 3 inactive subunits (dark gray) and one subunit that binds biotin with wild-type affinity (light gray). (**b**) Biotin binding site of wild-type streptavidin (from Protein Data Bank 1MK5)[11], highlighting the three residues mutated to create the 'dead' subunit (left). Asn23 and Ser45 were changed to alanines, removing two hydrogen bonds (dashed lines) to biotin, and Ser27 was changed to aspartate, to introduce a steric clash. In monovalent streptavidin, the residues mutated in the dead subunits are shown in green (right). (**c**) To make monovalent streptavidin, dead streptavidin subunits (D) and wild-type streptavidin subunits (A) in a 3:1 ratio were refolded from denaturant, giving a mix of streptavidin heterotetramers. Tetramers with a single 6His-tagged wild-type subunit were purified on a Ni-NTA column. (**d**) SDS-PAGE of chimeric streptavidins under nondenaturing conditions. Streptavidin with 4 dead subunits (D4), wild-type streptavidin with a 6His-tag (A4), the product of refolding of D and A in a 3:1 ratio (Mix), and chimeric tetramers with one (A1D3), two (A2D2) or three (A3D1) biotin binding subunits were loaded without boiling onto a polyacrylamide gel and visualized by Coomassie staining. (**e**) SDS-PAGE of chimeric streptavidins under denaturing conditions to break the tetramer into monomers.

generated a statistical mixture of tetramers of different composition. We purified the different tetramers using a nickel–nitrilotriacetic acid (Ni-NTA) column, eluting according to the number of 6His tags with increasing concentrations of imidazole. The tetramers could be distinguished by SDS-PAGE, if the samples were not boiled, according to the number of 6His tags present, showing that at least 30% of the initial mixture was of the monovalent A1D3 form (**Fig. 1d** and **Supplementary Methods**). Thus we obtained purified fractions of the monovalent A1D3 (final yield, 2 mg/l), as well as of the other chimeric streptavidins, A2D2 and A3D1. We confirmed the tetramer composition by boiling the samples before loading on SDS-PAGE, to determine the ratio of A to D subunits (**Fig. 1e**), and by electrospray ionization mass spectrometry (**Table 1** and **Supplementary Fig. 1** online). Despite the large mass of the streptavidin tetramer and the noncovalent interaction between subunits, we found good agreement between expected and observed masses for D4, A1D3, A2D2, A3D1 and A4.

Stability of monovalent streptavidin

We tested whether monovalent streptavidin would rearrange its subunit composition over time. We incubated A1D3 at 26 °C or at 37 °C and analyzed the reactions by SDS-PAGE to look for the appearance of D4 and A2D2 as a result of subunit exchange (**Fig. 2a**). No bands corresponding to D4 or A2D2 could be detected after incubation for up to one week. We did, however, detect a faint band, comprising ~3% of total protein and migrating faster than D4, after incubation at 26 °C for 1 week or at 37 °C for 1 d. This is most likely the result of a small degree of proteolysis of A1D3. Formation of A2D2 after incubation at 37 °C for 1 d was also not detected by silver staining, immunoblotting with an antibody to the 6His tag or mass spectrometry (data not shown). Thus substantial fractions of multivalent streptavidin will not be generated upon storage. We next tested the stability of A1D3 in terms of dissociation into monomers, as many mutations in the biotin binding site of streptavidin weaken tetramer stability[2]. We heated wild-type streptavidin and A1D3 in PBS at various temperatures for 3 min and determined tetramer disassembly by SDS-PAGE (**Fig. 2b**). A substantial fraction of A1D3 remained tetrameric even at 100 °C. There was little difference in thermostability between wild-type and monovalent streptavidin, suggesting that the mutations in D have minimal effect on the subunit interfaces, and that it should be possible to use A1D3 in assays requiring high temperatures.

Biotin binding by chimeric streptavidins

We used electrospray ionization mass spectrometry to determine the number of biotin molecules bound per tetramer. We acquired spectra of the different streptavidin tetramers with or without biotin. As expected, all four subunits of A4 were associated with biotin

Table 1 | Mass of different streptavidin tetramers with or without biotin

Tetramer	Predicted mass (without biotin)	Observed mass (without biotin)	Observed mass (with biotin)	Change in mass	Number of biotins bound
D4	52,962	52,997 ± 4	52,996 ± 12	−1 ± 2	0
A1D3	53,816	53,848 ± 5	54,088 ± 6	240 ± 4	1
A2D2	54,669	54,704 ± 6	55,193 ± 3	489 ± 4	2
A3D1	55,523	55,490 ± 45	56,201 ± 13	711 ± 39	3
A4	56,377	56,394 ± 8	57,378 ± 8	984 ± 7	4

The observed mass in daltons (±s.d.), determined by electrospray ionization mass spectrometry, is compared to the mass predicted from the sequence. From the change (±s.e.m.) upon addition of biotin (mass 244.31 Da), we determined how many biotin molecules were bound to each tetramer.

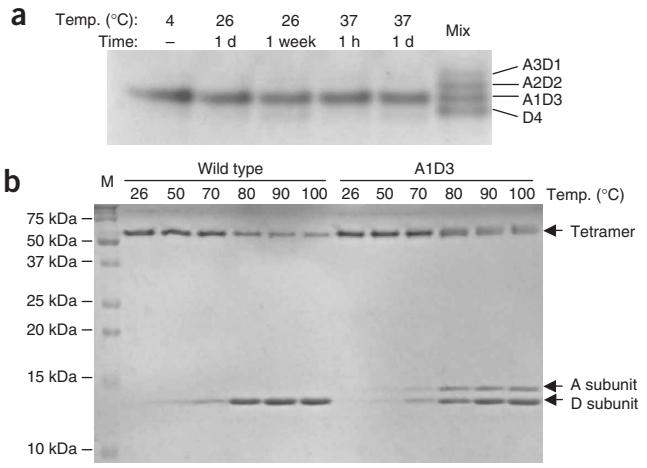

Figure 2 | Stability of monovalent streptavidin. (**a**) Stability to subunit exchange was determined by incubating 5 μM A1D3 in PBS as indicated, and detecting rearranged tetramers by 8% SDS-PAGE, by comparison to the initial product of refolding of D and A in a 3:1 ratio (Mix). (**b**) Stability of tetramer to heat denaturation was determined by incubating 5 μM wild-type streptavidin or A1D3 in PBS at the indicated temperatures for 3 min and then analyzing on 16% SDS-PAGE. M, marker.

(**Table 1** and **Supplementary Fig. 1**). No biotin binding by D4 could be detected. A1D3 was monovalent, binding a single biotin. The other chimeric tetramers bound one biotin per A subunit.

We determined the biotin binding affinity of the various streptavidin tetramers by measuring the competition with wild-type streptavidin for [^{3}H]biotin[9] (**Fig. 3**). The tetramer of dead subunits had negligible biotin binding, with an approximate K_d of $1.2 \times 10^{-3} \pm 0.2 \times 10^{-3}$ M (s.e.m.). The 6His tag did not affect biotin binding[9], as the measured affinity for A4 was $4.4 \times 10^{-14} \pm 1.1 \times 10^{-14}$ M (s.e.m.), similar to the untagged wild-type affinity of 4.0×10^{-14} M[1]. We determined a K_d of $4.8 \times 10^{-14} \pm 0.5 \times 10^{-14}$ M (s.e.m.) for monovalent A1D3 and a K_d of $5.4 \times 10^{-14} \pm 0.8 \times 10^{-14}$ M (s.e.m.) for divalent A2D2, indicating that the affinity of the chimeric tetramers for biotin was comparable to that of wild-type streptavidin.

We also evaluated the stability of biotin conjugate binding to A1D3 using an off-rate assay (**Fig. 3c**). As a positive control for biotin conjugate dissociation, we used a previously characterized streptavidin mutant with an accelerated off rate, S45A (ref. 11), and a streptavidin mutant that we found ourselves to have a fast off rate, T90I. The S45A and the T90I streptavidin mutants each showed >50% dissociation from a biotin-fluorescein conjugate in 1 h, whereas wild-type streptavidin and A1D3 both dissociated less than 10% in 12 h at 37 °C (**Fig. 3c**). A1D3 also had a comparable off rate to wild-type streptavidin for [^{3}H]biotin itself (**Fig. 3d**). The measured off rates were $5.2 \times 10^{-5} \pm 0.3 \times 10^{-5}$ s^{-1} (s.e.m.) for wild-type streptavidin and $6.1 \times 10^{-5} \pm 0.2 \times 10^{-5}$ s^{-1} (s.e.m.) for A1D3.

Labeling of cell-surface proteins without cross-linking

To test the use of monovalent streptavidin for labeling cell-surface proteins, we expressed cyan fluorescent protein (CFP) fused to a biotinylation sequence (the 'acceptor peptide'; AP) on the surface of HeLa cells using the transmembrane domain of platelet-derived

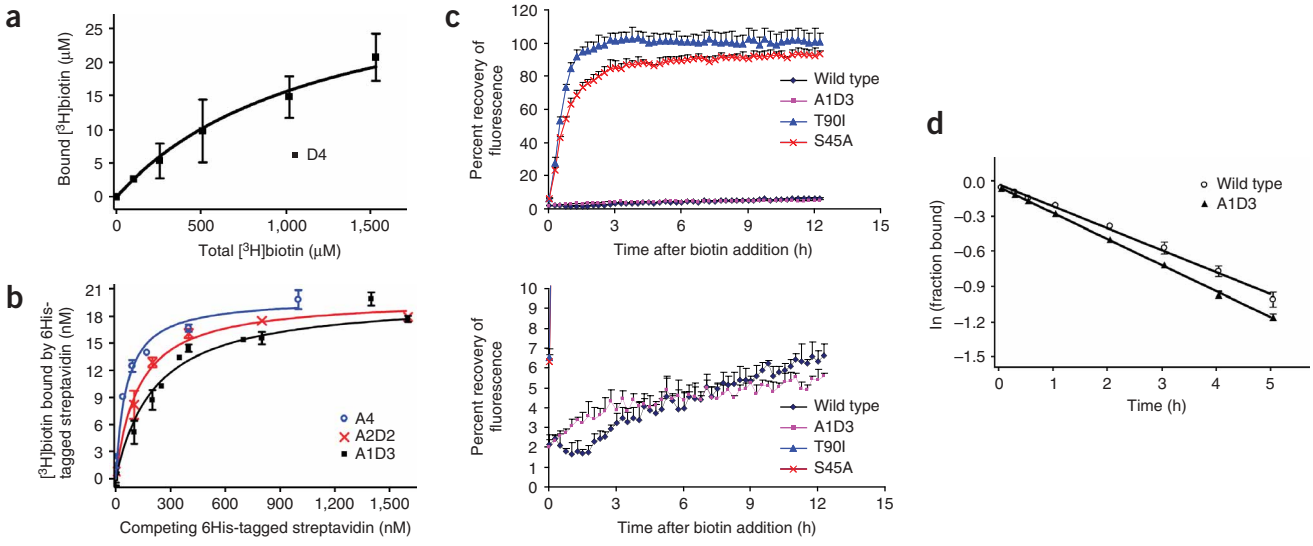

Figure 3 | Affinity and off rate of biotin binding to chimeric tetramers. (**a**) To determine the K_d for D4, 24 μM D4 was incubated with increasing concentrations of [^{3}H]biotin. After 20 h, the amount of bound [^{3}H]biotin was determined by precipitating D4. Means of triplicate measurements are shown ±1 s.d. Some error bars are too small to be visible. (**b**) To determine the K_d for A1D3, A2D2 and A4, increasing concentrations of A1D3, A2D2 or A4 were incubated with 20 nM [^{3}H]biotin and 50 nM wild-type streptavidin. After 20 h, chimeric tetramers were removed using Ni-NTA agarose, and the amount of [^{3}H]biotin bound to wild-type streptavidin in the supernatant was measured. From this value, the amount of [^{3}H]biotin bound to the chimeric tetramers was deduced. Means of triplicate measurements are shown ±1 s.d. (**c**) Off rate from a biotin conjugate. Wild-type, A1D3, S45A or T90I streptavidin was added in excess to biotin-4-fluorescein to quench its fluorescence. Excess competing biotin was added and fluorescence increase was monitored as biotin-4-fluorescein dissociated from streptavidin. The 100% value represents complete dissociation of biotin-4-fluorescein. Means of triplicate measurements are shown +1 s.d. The bottom panel is a magnification of the 0–10% region of the *y* axis, to illustrate the similar dissociation curves for wild-type streptavidin and A1D3. (**d**) Off rate from biotin. A1D3 or wild-type streptavidin was incubated with [^{3}H]biotin. Excess cold biotin was then added. After varying amounts of time at 37 °C, the amount of bound [^{3}H]biotin was determined by precipitating streptavidin. Means of triplicate measurement are shown ±1 s.d.

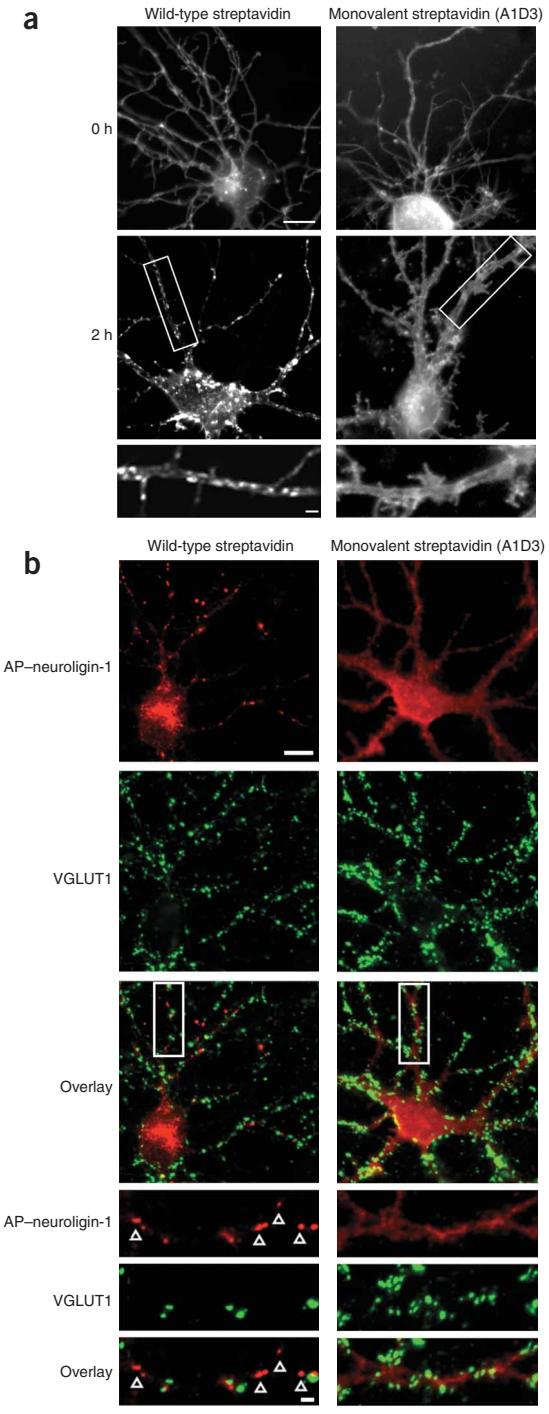

Figure 4 | Effect of monovalent and wild-type streptavidin on neuroligin-1 clustering. (**a**) Hippocampal neurons in dissociated culture were transfected with AP–neuroligin-1, biotinylated with biotin ligase and labeled with Alexa Fluor 568–conjugated wild-type (left) or A1D3 (right) streptavidin. After staining, cells were incubated for 0 h (top) or 2 h (middle) at 37 °C and streptavidin staining was visualized by live-cell fluorescence microscopy. Scale bar, 10 μm. Magnified images of the boxed regions are shown at the bottom. Scale bar, 1 μm. (**b**) Neurons were biotinylated and labeled with wild-type or A1D3 streptavidin as in **a**, incubated for 24 h and then stained for the presynaptic marker VGLUT1. Streptavidin (red) and VGLUT1 (green) signals are shown separately or overlaid. Scale bar, 10 μm. Magnified images of the boxed regions are shown below. Scale bar, 1 μm. Arrows indicate AP–neuroligin-1 clusters not apposed to presynaptic termini.

growth factor (PDGF) receptor (AP-CFP-TM)[12]. We biotinylated cell-surface acceptor peptide by incubation with extracellular biotin ligase and biotin-AMP for 10 min[7]. After washing the cells, we detected biotin with either wild-type or monovalent streptavidin, conjugated to Alexa Fluor 568. Detection was equally efficient and specific in both cases (**Supplementary Fig. 2** online). Incubating biotinylated cells with fluorescently labeled D4 tetramer gave no detectable staining, indicating that binding of A1D3 to biotinylated surface proteins should only occur through the A subunit.

We next tested whether labeling with monovalent streptavidin could prevent cross-linking of biotinylated cell-surface proteins, compared to wild-type streptavidin. Neuroligin-1 is a postsynaptic adhesion protein important in synapse formation[13]. Clustering of neuroligin-1 has been observed during synapse development and may depend upon interactions with the postsynaptic protein PSD-95 and the presynaptic binding partner neurexin[14,15]. We fused the extracellular N terminus of neuroligin-1 to an acceptor peptide tag and expressed this construct in dissociated hippocampal neurons. After adding biotin ligase to the cell medium and incubating for 5 min, we detected biotinylated AP–neuroligin-1 with either wild-type or monovalent streptavidin, conjugated to Alexa Fluor 568. Imaging immediately after labeling showed diffuse localization of AP–neuroligin-1 in both cases (**Fig. 4a**). After incubation for 2 h at 37 °C, however, AP–neuroligin-1 labeled with wild-type streptavidin was strikingly clustered, whereas AP–neuroligin-1 labeled with monovalent streptavidin was still predominantly diffuse (**Fig. 4a**, **Supplementary Fig. 3** and **Supplementary Table 1** online). Wild-type streptavidin also promoted AP–neuroligin-1 clustering if cells were incubated with streptavidin for 24 h rather than 2 h (**Fig. 4b**).

We next examined the effect of AP–neuroligin-1 clustering on neuron function. To assess the formation of excitatory synapses by these neurons, we stained the cells with an antibody against the presynaptic marker vesicular glutamate transporter-1 (VGLUT1) (**Fig. 4b**). By itself, transfection with a transgene encoding hemagglutinin (HA)-tagged neuroligin-1 (HA–neuroligin-1) will increase the number and size of VGLUT1 clusters[15] (**Supplementary Table 2** online). Transfection with the transgene encoding AP–neuroligin-1 caused the same increase in VGLUT1 clusters as HA–neuroligin-1, if we added wild-type streptavidin just before fixation (so that there was no time for neuroligin clustering to occur). If we labeled biotinylated AP–neuroligin-1 with monovalent streptavidin for 24 h, we observed a similar increase in the number and intensity of VGLUT1 clusters as for HA–neuroligin-1. This suggests that the acceptor peptide tag, biotin and monovalent streptavidin do not interfere with the presynaptic differentiation promoted by neuroligin. But our preliminary observations showed that when biotinylated AP–neuroligin-1 was labeled with wild-type streptavidin for 24 h, this led to a reduction in VGLUT1 cluster intensity (**Fig. 4b** and **Supplementary Table 2**). In addition, the AP–neuroligin-1 clusters observed after treatment with wild-type streptavidin were often isolated rather than colocalized with VGLUT1 clusters (colocalization 49 ± 0.3 %; **Fig. 4b**). These results suggest that synapse formation can be disrupted by wild-type streptavidin labeling, a finding consistent with previous work indicating that artificial aggregation reduces the overall number of functional synapses formed[14,16]. The use of monovalent streptavidin, in contrast, can provide stable and specific labeling of neuroligin-1, without perturbing protein function through cross-linking.

DISCUSSION

We have generated a monovalent streptavidin, by making a chimeric streptavidin tetramer with a single active biotin binding subunit. This monovalent streptavidin bound to biotin with similar affinity and stability as wild-type streptavidin, did not rearrange its subunits over time and had high thermostability. Monovalent streptavidin should permit one to make use of its femtomolar binding affinity without additional unwanted multimerization.

Aside from illustrating how monovalent streptavidin can be used for stable and cross-linking–free protein detection in cellular imaging experiments, our experiments with neuroligin-1 demonstrate how engineered streptavidins can be used to examine the biological consequences of controlled clustering of cellular proteins. Cross-linking is used by cells to regulate protein activity, mobility and interactions, as demonstrated for many growth factor receptors and transcription factors[8]. Here we only compared monovalent and tetravalent (wild-type) streptavidins, but it would also be possible to use the entire series of purified streptavidin heterotetramers to examine the functional effects of receptor dimerization, trimerization and tetramerization.

The many alternative elegant approaches to site-specific labeling of proteins in living cells cannot all be described here, but have recently been reviewed[17–19]. The key point is that no single labeling approach is ideal in every circumstance. For example, fluorescent proteins or biarsenical labeling of tetracysteine tags[20] are valuable methods that do not suffer from probe dissociation or cause cross-linking, but they are limited for single-particle tracking by the moderate brightness and photobleaching of their fluorophores. Biarsenical labeling also cannot be used to label proteins specifically at the cell surface, although it has the advantage over our method that it can be used to label intracellular proteins. The advantages of labeling by means of site-specific biotinylation and monovalent streptavidin, as compared to antibody labeling, are twofold. First, the biotin-streptavidin interaction provides a much more stable linkage that permits long-term imaging over hours or even days. This strong interaction also eliminates the concern that the label might rapidly dissociate and reassociate with different cell-surface proteins over the time course of the imaging experiment, complicating tracking measurements. Second, antibodies are bivalent and can cross-link target proteins, as wild-type streptavidin does. Fab antibody fragments bind monovalently, but usually have substantially lower effective affinities, compounding the problem of antibody-epitope instability. Conversely, when high-affinity antibodies are available, antibodies have the advantage that they can be used to detect endogenous proteins, avoiding possible artifacts from transgene overexpression.

The need to purify different chimeric forms of streptavidin, as previously performed to improve reversibility[21,22] and as shown here, could be avoided if the four subunits could be genetically joined to make a single-chain streptavidin. But the large distance between the termini of streptavidin means that long linkers would be required, which are likely to impair folding. Attempts to circumvent this problem by circularly permuting streptavidin have yielded forms with $K_d > 10^{-8}$ M[23,24]. A circularly permuted tetravalent single-chain avidin with wild-type binding affinity was recently reported[25]. It will be valuable to generate an analogous single-chain tetravalent streptavidin and then to make it monovalent using the approach presented here, as avidin binds ~40-fold less tightly to biotin conjugates than streptavidin[26].

Bottom-up nanotechnology refers to the emerging use of self-assembly to construct multimolecular assemblies on the nanometer scale[27]. Streptavidin is one of the most common bridges in such assemblies, linking biotinylated DNA molecules, proteins and inorganic structures including carbon nanotubes and gold particles[27]. Such streptavidin bridges are either four-way junctions, if the biotinylated ligand is used at a saturating concentration, or statistical mixtures of zero- to four-way junctions, if the biotinylated ligand is used at a subsaturating concentration. The use of the chimeric streptavidin tetramers described in this paper should permit one to select whether a one-, two-, three- or four-way junction is generated. Poor yields of the desired junction, resulting from using a statistical mixture, are tolerable if there is only one step in assembly, but give unacceptable final yields if there are multiple steps in the assembly. This limits the complexity of the nanostructure that can be assembled. Thus chimeric streptavidins should be valuable building blocks for the construction of novel microarrayed sensors[28] and microelectronic circuits[29].

METHODS

Streptavidin expression and purification. We induced *E. coli* BL21(DE3) cells transformed with the streptavidin-pET21a expression plasmid (GenBank DQ376186), at OD_{600} 0.9 with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After incubating the cells for an additional 4 h at 37 °C, we purified inclusion bodies from the cell pellet using B-PER (Pierce), following manufacturer's instructions, and dissolved them in 6 M guanidinium hydrochloride (GuHCl; pH 1.5). To generate chimeric streptavidins, we estimated the concentration of each unfolded subunit from OD_{280} values in GuHCl and mixed the subunits in the desired ratio. We refolded the subunits in GuHCl by rapid dilution into PBS and concentrated them by ammonium sulfate precipitation, as described[30]. We redissolved the precipitate in PBS and dialyzed it 3× against PBS. This step was sufficient to purify A4, D4 and wild-type streptavidin. To purify chimeric streptavidins, we loaded a Poly-Prep column (Bio-Rad) with 1.8 ml Ni-NTA agarose (Qiagen) and washed it with 8 ml binding buffer (50 mM Tris-HCl, 300 mM NaCl; pH 7.8), using gravity flow at 26 °C. We diluted the streptavidin in PBS two-fold in binding buffer and loaded it onto the column. We washed the column with 8 ml washing buffer (binding buffer with 10 mM imidazole), eluting D4. Then we added 5 ml elution buffer 1 (binding buffer with 70 mM imidazole) and eluted A1D3, with some A2D2 eluted in later fractions. We collected 0.5-ml fractions of this elution, and of subsequent elutions with 5 ml elution buffer 2 (binding buffer with 100 mM imidazole), eluting principally A2D2, and then 5 ml elution buffer 3 (binding buffer with 125 mM imidazole), eluting principally A3D1. We mixed samples of each fraction with SDS loading buffer and then loaded the samples without boiling onto 8% SDS–polyacrylamide gels. We pooled and dialyzed fractions of the correct composition, determined by comparison to bands from the initial refold, in PBS. When required, we concentrated samples using a Centricon Ultracel YM10 (Millipore).

Cell culture, biotinylation and imaging. HeLa stably expressing AP-CFP-TM or Ala-CFP-TM have been previously described[7]. We prepared dissociated primary neuronal cultures from E18/19 rats and transfected them with transgenes encoding HA–neuroligin-1,

AP–neuroligin-1 or Ala–neuroligin-1 using Lipofectamine 2000 at division (DIV) 6 (ref. 15). All animal experiments were performed in accordance with the University of British Columbia Animal Care Committee.

We performed enzymatic biotinylation and imaging of HeLa transfectants as previously described[7], except instead of 10 μM biotin and 1 mM ATP, we added 10 μM biotin-AMP to give equivalent biotinylation (data not shown) while minimizing the risk of purinoreceptor activation by ATP. We biotinylated HeLa transfectants for 10 min at 26 °C, and stained with 10 μg/ml Alexa Fluor 568–conjugated wild-type streptavidin, D4 or A1D3 for 10 min at 4 °C. We biotinylated neurons at DIV 8 in Hanks' balanced salt solution (HBSS; Invitrogen) with 0.2 μM biotin ligase and 10 μM biotin-AMP for 5 min at 37 °C. We then washed neurons with HBSS and incubated for 2 min with 5 μg/ml Alexa Fluor 568–conjugated wild-type streptavidin (Molecular Probes) or A1D3 at 37 °C. After washing with NeuroBasal medium (Invitrogen) supplemented with 2% B-27 (Invitrogen), 50 U/ml penicillin, 50 μg/ml streptomycin and 0.2 mM L-glutamine, we incubated the neurons in the same medium for 0–2 h at 37 °C. Then we imaged the cells immediately or fixed them in –20 °C methanol. We did not observe labeling by wild-type streptavidin on neurons transfected with Ala–neuroligin-1 containing a point mutation in the acceptor peptide (data not shown), demonstrating the specificity of labeling[7].

To analyze excitatory synapses, we biotinylated the cells and stained them with streptavidin as above; we repeated biotinylation and streptavidin staining at 6 h, and incubated the cells for an additional 18 h before fixation in –20 °C methanol[15]. We stained samples for VGLUT1 using guinea pig anti-VGLUT1 (1:1,000; Chemicon) and then by goat anti-guinea pig Alexa Fluor 488 (1:1,000; Molecular Probes), both in blocking solution (PBS with 0.3% Triton X-100 and 2% normal goat serum; Vector Laboratories) for 1 h at 26 °C or overnight at 4 °C.

We collected images of HeLa cells on a Zeiss Axiovert 200M inverted epifluorescence microscope using a 40× oil-immersion lens and a MicroMAX charge-coupled device (CCD) camera (Roper Scientific). We collected CFP (420DF20 excitation, 450DRLP dichroic, 475DF40 emission) and Alexa Fluor 568 (560DF20 excitation, 585DRLP dichroic, 605DF30 emission) images and analyzed them using OpenLab software (Improvision). Fluorescence images were background-corrected. We acquired neuron images for 500–800 ms on a Zeiss Axiovert 200M microscope with a 63× 1.4 numerical aperture Acromat oil immersion lens and a monochrome 14-bit Zeiss Axiocam HR CCD camera with 1,300 × 1,030 pixels. In every case we prepared and imaged wild-type and A1D3 streptavidin samples under identical conditions. To correct for out-of-focus clusters within the field of view, we acquired focal-plane z stacks and performed maximum intensity projections off-line. Images were scaled to 16 bits and analyzed in Northern Eclipse (Empix Imaging) with user-written software. Briefly, images were processed at a constant threshold level (of 32,000 pixel values) to create a binary mask image, which was multiplied with the original image using Boolean image arithmetic. The resulting image contained a discrete number of clusters with pixel values of the original image. Only dendritic streptavidin clusters greater than 5 pixels in size and with an average pixel values two times greater than background pixel values were used for analysis. Results were then calculated in terms of clusters per micrometer of dendrite. For assessment of presynaptic termini, we used VGLUT1 clusters larger than 10 pixels for analysis, in which the average gray levels and number of clusters per micrometer were compared between transfected dendrites and untransfected dendrites within the same field of view. Colocalization of wild-type streptavidin clusters with VGLUT1 clusters was determined by Boolean image arithmetic from their binary mask images. Only clusters with a two-pixel overlap were counted as colocalization of pre- and postsynaptic termini. We performed the two-tailed parametric Student's *t*-test to calculate statistical significance of results between experimental groups. '*n*' represents the number of transfected neurons for which clusters were measured.

Additional methods. Descriptions of plasmid construction, fluorophore labeling of streptavidin, mass spectrometry and measurements of streptavidin K_d, off rate and thermostability are available in **Supplementary Methods**.

Note: Supplementary information is available on the Nature Methods website.

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AUTHORS' CONTRIBUTIONS STATEMENT

M.H., D.J.-F.C., K.G. and P.C.D. performed the experiments. M.R.G. produced new reagents for the paper. M.H., A.E.-H., N.L.K. and A.Y.T. designed the research. M.H. and A.Y.T. wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Methods* website for details).

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