Profiling surface proteins on individual exosomes using a proximity barcoding assay

Di Wu¹,²,³, Junhong Yan¹, Xia Shen⁴,⁵,⁶, Yu Sun⁷,¹¹, Måns Thulin⁸,¹², Yanling Cai⁹, Lotta Wik¹, Qiujin Shen¹, Johan Oelrich³, Xiaoyan Qian², K. Louise Dubois¹⁰, K. Göran Ronquist¹⁰, Mats Nilsson², Ulf Landegren¹ & Masood Kamali-Moghaddam¹

Exosomes have been implicated in numerous biological processes, and they may serve as important disease markers. Surface proteins on exosomes carry information about their tissues of origin. Because of the heterogeneity of exosomes it is desirable to investigate them individually, but this has so far remained impractical. Here, we demonstrate a proximity-dependent barcoding assay to profile surface proteins of individual exosomes using antibody-DNA conjugates and next-generation sequencing. We first validate the method using artificial streptavidin-oligonucleotide complexes, followed by analysis of the variable composition of surface proteins on individual exosomes, derived from human body fluids or cell culture media. Exosomes from different sources are characterized by the presence of specific combinations of surface proteins and their abundance, allowing exosomes to be separately quantified in mixed samples to serve as markers for tissue-specific engagement in disease.
Exosomes are a subclass of membrane-coated extracellular vesicles with sizes of 30–100 nm, which are released from cells by exocytosis. Exosomes are found in most body fluids, and they have been shown to play key roles in processes such as coagulation, intercellular signaling, immune responses, and cellular waste management. Compelling evidence suggests that exosomes may have a role in the spread of cancer from a primary tumor to metastasis sites, and they are promising as tissue-specific biomarkers for liquid biopsy. Exosomes are highly heterogeneous in molecular composition, and their surface proteins bear characteristics of their tissues of origin, rendering specific subclasses of these vesicles promising to demonstrate pathology affecting specific tissues. Accordingly, it is important to investigate exosomes individually as such information may be lost in bulk-level analyses.

Recently, new technologies have emerged that improve opportunities for detection of exosomes. Imaging flow cytometry overcomes obstacles in traditional flow cytometry by including a CCD camera with a 60× objective, allowing detection of vesicles with sizes below 500 nm through enhanced fluorescence. However, only a small number of fluorophore-labeled antibodies can be resolved in this way. Nano-plasmonic sensors utilize sophisticated nanohole arrays to first isolate single exosomes via specific capture antibodies, followed by protein profiling using detection antibodies. The combination of capture and detection antibodies limits the analysis to two protein targets per exosome, and sandwich immune assays are limited to analyzing pairs of proteins on exosomes in bulk. Therefore, methods are needed to more comprehensively profile proteins in high multiplex for individual exosomes.

DNA-assisted immunoassays combine affinity probes with conjugated amplifiable oligonucleotides, converting protein identities to DNA sequences for protein detection even at the level of single molecules or molecular complexes. In particular, proximity ligation or extension assays can offer improved specificity of analysis and confer information about protein compositions via ligation or extension of pairs of DNA strands brought in proximity via their conjugated antibodies. A multiple-recognition proximity ligation assay (4PLA) has been developed where exosomes are captured by an immobilized antibody, whereupon four antibody-DNA conjugates give rise to amplifiable DNA strands for highly specific and sensitive detection of prostate-derived exosomes—prostatomes. Similarly, antibody-DNA conjugates have been used for flow-cytometric detection of individual exosomes by pairwise ligation of several sets of antibody-DNA conjugates enhanced via rolling circle amplification (RCA) for fluorescence detection. However, new technologies are required to survey higher orders of protein species on large sets of exosomes in parallel in order to assess their heterogeneity.

Here, we report a proximity-dependent barcoding assay (PBA), as a high-throughput approach to simultaneously profile 38 surface proteins for their presence on individual exosomes. In PBA, we use micrometer-sized single-stranded DNA clusters, each having hundreds of copies of a unique DNA motif, generated via RCA, to barcode individual exosomes. The protein composition on the surface of individual exosome is converted to DNA sequence information via bound antibody-DNA conjugates that incorporate a random tag sequence repeated in each RCA product. After amplification by PCR, information about protein and exosome identity brought together in DNA strands is decoded by next-generation sequencing to identify the surface protein composition of individual exosomes.

Results
Design and workflow of PBA. PBA probes were prepared by conjugating antibodies with DNA oligonucleotides comprising a 8-nucleotide (nt) proteinTag that served to identify the target exosomal surface protein, and a 8-nt random unique molecular identifier (UMI) sequence, here referred to as a molecule tag (moleculeTag), to distinguish individual protein molecules after PCR amplification (Fig. 1a). Reagents to barcode individual exosomes were prepared by RCA of circularized DNA molecules containing a 15-nt random DNA sequence (complexTag). Each RCA product includes several hundred identical copies of a unique complexTag (Fig. 1b), and in the PBA procedure these become incorporated in the antibody-conjugated oligonucleotides, and serve to identify proteins on individual exosomes having become colocalized with unique RCA products as described in Fig. 1d.

In PBA, exosomes are mixed with PBA probes in solution before being captured sparsely in 96-well microtiter wells coated with cholera toxin subunit B (CTB) that binds GM1 gangliosides in the exosome membranes, followed by washes to remove free antibody conjugates. Diluted RCA products are then added, allowing individual RCA products, similar in size to exosomes, to interact with single exosomes by having their bound PBA probes hybridize to the RCA products. PBA probes bound to the same exosome are brought in proximity, and incorporate the same complexTag from a nearby RCA product by DNA polymerase-mediated extension (Fig. 1c). Next, the extension products are amplified for preparation of a sequencing library. Neither oligonucleotides on exosomes that have failed to encounter an RCA product nor isolated RCA products can give rise to amplifiable products. After sequencing amplification products, the reads of each sample are sorted by complexTags and proteinTags to identify participating proteins on individual exosomes. By counting the total number of different moleculeTags for each of the proteinTags sharing the same complexTag, all detected protein molecules from individual exosomes in a sample can be identified and quantified.

Validation of PBA using a separate or mixed incubation system. To ascertain that PBA could correctly identify members of protein complexes, we first prepared artificial complexes by allowing streptavidin (STV) to bind combinations of biotinylated oligonucleotides (Fig. 2a). We incubated STV with oligonucleotides carrying each one of four proteinTags in four separate preparations, such that each STV in the mixture bound oligonucleotides with the same proteinTag before these four STV preparations were combined (separate incubation). If two different STV-oligonucleotide complexes would be erroneously identified by PBA as a single one, then there is an ~75% possibility that such falsely identified complexes would involve more than one proteinTag. For comparison, we also incubated STV with a mixture of all four biotinylated oligonucleotides, such that each STV would bind some combination of these four oligonucleotides (mixed incubation). STV were incubated with biotinylated oligonucleotides at either a 1:10 or 10:1 molar ratios. In the analysis of samples from separate preparations, less than 1% of the recorded complexes included more than one proteinTag. In the mixed incubation samples with oligonucleotides in excess, 21.0% of the complexes were shown to include two distinct proteinTags, while 2.4% and 0.2% of complexes had three or four proteinTags, respectively. As expected, we observed more complexes with more than one oligonucleotide per STV in preparations where oligonucleotides were in excess of STV. In these preparations, the recorded complexes containing 2, 3, or 4 moleculeTags from either separate or mixed preparations were sorted according to the proteinTags per complex (Fig. 2b). The majority of the separately prepared complexes contained only 1
proteinTag, while the majority of complexes prepared with mixes of the four oligonucleotides contained more than one proteinTag, with the distribution close to theoretical values. The results indicate that under the appropriate conditions PBA can be used to profile individual protein complexes quantitatively, with minimal risk that proteins from separate complexes are erroneously clustered together (Supplementary note 1, Supplementary Figs. 3–5). Having established that PBA can identify members of complexes in the streptavidin model system, we next investigated the performance of the method for profiling surface proteins on individual exosomes. We prepared four PBA probes by conjugating CD9 antibodies to two distinct oligonucleotides, forming PBA probes with either proteinTag A (CD9-TagA) or proteinTag B (CD9-TagB), and conjugating CD63 antibody with two other oligonucleotides to obtain either proteinTag C (CD63-TagC) or proteinTag D (CD63-TagD). We then mixed antiCD9-TagA and antiCD63-TagC as probe Set1, and antiCD9-TagB and antiCD63-TagD as the second probe Set2. Figure 3a presents results from an experiment where exosomes isolated from the K562 cell line were incubated separately with the two sets of PBA probes before these were pooled and subjected to PBA with capture in microwells.
annealing to RCA products, extension and sequencing library preparation. In a parallel reaction (Fig. 3b), the exosomes were incubated with all four PBA probes, followed by the PBA workflow. In the reaction with separate probe sets, we observed less than 5% of exosomes with PBA probes coming from two different sets (CD9-TagA with CD9-TagB, CD63-TagC with CD63-TagD, CD9-TagA with CD63-TagD, or CD9-TagB with CD63-TagC), while a large majority of the exosomes were tagged with both CD9 and CD63 from the same probe set (CD9-TagA and CD63-TagC or CD9-TagB and CD63-TagD; Fig. 3a). By contrast, in the reaction with mixed probe sets, exosomes were tagged with probes from both probe sets (Fig. 3b). This again demonstrated the ability of PBA to analyze individual exosomes with minimal risk of false identification, important for characterizing populations of exosomes in biological samples, as a basis to evaluate their distinct diagnostic potential.

**Fig. 2** Validation of PBA using STV-biotin-oligonucleotide complexes. Tetrameric streptavidin molecules were incubated with four different biotinylated oligonucleotides, either separately or in a mixture of all four oligonucleotides. Streptavidin and biotinylated oligonucleotides were combined at molar ratios of 1:10 or 10:1. **a** The numbers of observed complexes with different numbers of proteinTags and moleculeTags are summarized in pie charts. **b** When the oligonucleotides were in excess, the numbers of complexes from separate or mixed oligonucleotides incubation with 2, 3, or 4 moleculeTags were grouped according to the number of proteinTags per complex. Theoretical ratios were calculated for the mixed sample.
been reported to be relevant for cancer metastasis\textsuperscript{2}.

lines, with a particular focus on integrin markers, which have
markers, previously used to analyze exosomes from cancer cell
bodies were chosen to include many known exosomal cancer
sample was individually exposed to the PBA probes. The anti-
SKNSH, originating from different tissues (see Methods). Each
K562, KatoIII, MNK45 MNK7, AGS, MM1, DAUD1, BPH-1,

from serum from healthy donors (serum exosomes) and one from
semenal fluid, referred to as prostasomes, which have been sug-
gested to serve as biomarkers for prostate cancer\textsuperscript{12}. Exosomes
were also prepared from conditioned media of the 16 human cell
lines\textsuperscript{10}, BLC21, U87MG, A549, HCT116, PC3, HEK293, COLO1,
K562, KatoIII, MNK45 MNK7, AGS, MM1, DAUD1, BPH-1,
SKNSH, originating from different tissues (see Methods). Each
sample was individually exposed to the PBA probes. The anti-
odies were chosen to include many known exosomal cancer
markers, previously used to analyze exosomes from cancer cell
lines, with a particular focus on integrin markers, which have
been reported to be relevant for cancer metastasis\textsuperscript{2}.

By counting the total moleculeTags connected with each
protein for each sample, the total protein abundance can be
recorded (Supplementary Fig. 7). By comparing the deduced surface protein pro-
files from individual prostasomes or from exosomes from the K562 cell line
with those found in serum, we identified combinations of protein
species found mainly in prostasomes (>99%) or in K562
exosomes (>95%) (Fig. 5a). We used this information to mimic
analysis of heterogeneous samples, prepared by spiking in
exosomes from a single sample source. Lastly, we investigated
exosomes for which three or more proteins had been identified
\((n = 61882)\). Here, exosomes from the same sample sources were
seen to colocalize by t-SNE analysis. For some regions, exemplified by dotted circles in Fig. 4b, exosomes from particular
sample sources are seen to dominate. This demonstrates that
exosomes from different sources are better distinguished when
more surface proteins are identified. The PBA analysis does not
necessarily detect all proteins present on a given exosomes
because of less than quantitative antibody binding and limited
sequencing depth.

By comparing the deduced surface protein profiles from
individual prostasomes or from exosomes from the K562 cell line
with those found in serum, we identified combinations of protein
species found mainly in prostasomes (>99%) or in K562
exosomes (>95%) (Fig. 5a). We used this information to mimic
analysis of heterogeneous samples, prepared by spiking in
different amounts of either purified prostasomes or K562
exosomes or both in a fixed amount of serum exosomes. PBA
analysis revealed the expected correlation between proportions of
added and recorded exosomes with prostasome- or K562-
selective combinations of PBA probes (Fig. 5b–c). Duplicate
measurements demonstrated that the measured proportions were
reproducible (Supplementary Fig. 6). Pairs of antibodies binding
prostasomes as revealed by PBA were also used as probes in solid-
phase proximity ligation assays (SP-PLA) providing efficient
quantitation (Supplementary Fig. 7).
**Discussion**

The representation of different classes of exosomes in biological samples may confer valuable diagnostic information, but this information is not readily accessible by current methods for exosome analysis. In biomarker discovery, exosome species that might be of diagnostic value for disease could be present in low abundance among other exosome populations in a sample, obscuring any diagnostic value. Here, we have used DNA sequencing to decode combinations of antibodies with protein-specific DNA tags (proteinTags), binding individual exosomes, by

---

**Fig. 4** Bulk protein counts and visualization of individual exosomes. **a, b** Exosomes from 18 different samples were analyzed for the presence of 38 proteins (40 proteinTags) by PBA. **a** Heatmaps representing the total amounts of the proteins by log(moleculeTag + 1) found on exosomes from different sources. **b** Individual exosomes with one identified protein type, two protein types, and three or more protein types were visualized by t-SNE according to their protein compositions, with color and shape of the symbols representing the source of each exosome. Cells from two regions dominated by exosomes from the cell lines AGS and BPH-1 are highlighted in circles with dotted contours.
Fig. 5 Quantification of exosomes from different sources according to their surface protein combinations, as revealed by PBA. a Protein combinations selective for either K562 exosomes (orange) or prostasomes (blue), compared to exosomes from serum (gray), were sorted based on the number of exosomes with specific combinations. Here, the top 50 for each exosome are displayed. b, c Serial dilution of K562 exosomes or prostasomes spiked in serum exosomes were quantified using K562- or prostasomes-selective combinations, respectively. The exosomes identified with either K562- or prostasome-selective combinations are indicated by orange and blue filled circles in the tSNE plots in b, and with orange and blue bars, respectively, in the bar plots in c. The sizes of the colored circles in (b) are proportional to the number of exosomes with the same protein combination. Each two vertically adjacent tSNE plots and two horizontally adjacent bars are illustrating two replicates.
associating them via DNA extension with single members of a large repertoire of DNA bundles—RCA products—with multiple copies of a particular random DNA tag—the complexTag. We have used the assays to identify the protein composition of individual exosomes. PBA can identify and quantify large numbers of combinations of proteins from a given set on individual exosomes or any other clusters of proteins.

PBA is performed in microtiter wells without the need for compartmentalization in nanowell® or droplets25–27 or the use of special equipment, by using large numbers of submicrometer-sized DNA bundles generated by RCA, similar in size to the GC content of special equipment, by using large numbers of submicrometer-sized oligonucleotides complexes were combined into one, and both this pool and the

*Antibodies and oligonucleotides.* The antibody preparation directed against streptavidin was purchased from Thermo Scientific® (S1014). The sources of antibodies directed against surface proteins on exosomes are summarized in Supplementary Table 2. The oligonucleotides used in this study were purchased from IDT or Solulink (Supplementary Table 1).

**Preparation of antibody-oligonucleotide conjugates (PBA probes).** The conjugation of oligonucleotides to antibodies (Supplementary table 2) was performed as follows: Twenty µg of each antibody was activated by adding 1 µl of 4 mM Sulfo-NHS-EDMA-NHS (Thermo Scientific®) in 100 mM sodium bicarbonate (pH 8.5), and incubating at room temperature (RT) for 2 h. After 1 h of antibody activation, 3 µl of each thiol-modified oligonucleotide at a concentration of 100 µM was reduced by adding 12 µl of 100 mM DTT (Sigma–Aldrich) in 1x PBS with 5 mM EDTA, and incubating at 37 °C for 1 h. The activated antibodies and reduced oligonucleotides were separately purified using Zebra Spin Desalting Plates, 7 K MWCO (Thermo Scientific®) according to the manufacturer’s recommended procedure. Each purified antibody was then mixed with one type of oligonucleotide (Supplementary Table 1), and directly followed by dialysis in a Slide-A-Lyzer Mini Dialysis Device, 7 K MWCO, 0.1 ml (Thermo Scientific®) against PBS containing 0.01 U/µl of bovine serum albumin (BSA) at 4 °C. The conjugates were stored at µM antibody concentration in PBS with 0.1% BSA at 4 °C.

**Preparation of RCA products.** Padlock oligonucleotides, including a 15 nt random sequence (100 nM) were ligated into circular DNA strands in the presence of template (100 nM) in 1x phz29 buffer (Thermo Scientific®), 10 mM Mg-acetate, 66 mM K-acetate, 0.1% (v/v) Tween 20, 0.1 mM DTT) containing 0.01 U/µl T4 ligase (New England Biolabs®), and 1 μM probe (Thermo Scientific®). The ligation was performed at 37 °C for 30 min. To remove unligated padlock oligonucleotides, exonuclease 1 and exonuclease III (Thermo Scientific®) were added to the ligation mix to a concentration of 0.2 U/µl and 2 U/µl, respectively. The reactions were incubated at 37 °C for 30 min and terminated by incubation at 85 °C for 20 min. Then the RCA primer (the same oligonucleotide used as ligation template) was adjusted to a concentration of 100 nM and incubated at 37 °C for 20 min to reanneal to the circular DNA strands. The RCA reactions were initiated by adding d(A, G, C, T)P at concentrations of 1 mM and phi29 polymerase (Thermo Scientific®) at 0.1 U/µl. RCA was performed at 37 °C for 10 min, and terminated by heating at 65 °C for 10 min. The RCA products were kept at −20 °C until use. The RCA products were characterized with Nanoparticle Tracking Analysis NTa (Malvern Nanosight NS300) according to the manufacturer’s instructions.

**Capturing STV-biotin oligonucleotide complexes.** For each reaction, 200 ng anti-STV antibodies, diluted in 50 µl 100 mM carbonate buffer (pH 9.6), was added to 8–well RoboStrip (847–0501000103, Analytik Jena) and incubated overnight at 4 °C. After two washes with 100 µl washing buffer (1x PBS with 0.05% Tween20 (Sigma–Aldrich)), 50 µl blocking buffer (1x PBS with 1% BSA (Sigma–Aldrich)) was added and the plates were incubated at 37 °C for 1 h. After removing the blocking buffer, pre- assembled STV- oligonucleotide complexes were diluted to 10 pM (according to the concentration of STV) in 20 µl washing buffer, and added to the antibody-immobilized microplates, and incubated at 1 h at RT.

**Probing and capturing exosomes.** For each reaction, 50 ng biotinylated CBT (C-34779, Thermo Scientific®) was diluted in 25 µl PBS and incubated overnight in STV-coated PCR tubes (PGBSTF-SAS/100, Biomat). The wells in the plates were then washed twice with 100 µl washing buffer. Exosomes from each sample source or cell line were mixed with all antibody conjugates (20 nM for each), directed against 10 different antibodies (PBS, 0.05% Tween20, 1 mg/ml salmon sperm DNA (1563201, ThermoFisher Scientific®) and 1% BSA) at 4 °C overnight. After incubation, 1 µl of the exosome-antibody conjugate complexes

 Bárány, A., Gábor-Szirmai, F., Sebestyén, Z. et al. Capture of specific exosomal RNAs from heterogeneous samples using a unique nanobead-based assay. *Nature Commun.* 11, 1211 (2020). https://doi.org/10.1038/s41467-020-13384-1
were diluted in 25 μl PBA buffer, and 20 μl was incubated in wells coated with biotinylated CTB for 15 min at RT. For replicate measurements, 2 μl of exosomes were diluted in 50 μl PBA buffer, and 20 μl were added into two separated wells.

Proximity barcoding. The captured STV-oligonucleotide complexes or exosomes with antibody conjugates were washed twice with 100 μl washing buffer. Then 25 μl of the RCA products at a concentration of 1 nM and 500 nM blocking oligonucleotide was added to the reaction wells and incubated at 37 °C for 15 min. The blocking oligonucleotide extending to adjacent copies of monomers in the RCA products containing the complexTags. After two washes with 100 μl washing buffer, 25 μl of T4 DNA polymerase buffer containing 1.25 unit of T4 DNA polymerase and 100 μM d(A, T, G, C)/TP was added and the reaction mix was incubated at RT for 15 min.

Amplification of complex-tagged extension products from the antibody-conjugated oligonucleotides. The reaction mix was washed and 30 μl of PCR mix containing 1X Phusion HF Buffer (2 mM MgCl2, 0.2 mM dATP, dGTP, 1X SyBR Green I, 1% DMSO, 500 nM PCR primers (pbw- and pba-rev), 0.02 U/μl Phusion polymerase and 0.02 U/μl Uralci-DNA glycosylase (all from Thermofisher Scientific) was added to each reaction well. The plate was then transferred to a thermal cycler (MX3005, Stratagene) for qPCR with an initial incubation of 37 °C for 1 min, 95 °C for 2 min, followed by 30 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s.

Sample indexing and library preparation for sequencing. After PCR amplification of extension products from PBA reactions, 1 μl of the amplification reaction was spiked in 10 μl of index PCR mix containing 10 μM each of fwd-index and rev-index primer pairs, and the reactions were incubated for an initial 2 min at 95 °C, followed by 20 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The indexed PCR products were diluted 20 times into new PCR mixes containing 1X Phusion HF Buffer (Thermofisher Scientific), 0.2 mM dATP, dGTP, 1% SyBR Green I (Thermofisher Scientific), 1X SyBR Green I (Thermofisher Scientific), 1% DMSO, 500 nM PCR primers (library-fwd and library-rev) and programmed for an initial 2 min at 95 °C, and 15 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The PCR products were pooled and purified using QIAquick PCR Purification Kit. The purified DNA was sequenced using MiSeq Reagent Kit v2, 300 cycles by MiSeq or NextSeq Reagent Kit 75SE, NextSeq (Illumina).

SP-PLA for prostate-specific detection in 10% human plasma. Biotinylated CD26 and CD59 were prepared using ChromaLink Biotin Labeling Reagent (Solulink B-SP-PLA for prostasome detection in 10% human plasma and CD59 were prepared using ChromaLink Biotin Labeling Reagent (Solulink B-SP-PLA for prostasome detection in 10% human plasma.

Data analysis. The BCL files for each sample were converted to fastq formats by using bcl2fastq (Illumina) with pair indexes. Then the complexTag, proteinTag, and moleculeTag were extracted from three fixed segments within each read. Reads with only one count were removed from the analysis. The tags were then sequenced to the annotated genome to obtain complexTag, proteinTag, and moleculeTag using an in-house developed Perl script. The number of exosomes with given combinations of proteins were calculated using an in-house developed R script. The t-SNE algorithm was applied was implemented in the R package Rtsne, and all the parameters were set to default in our pipeline.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

References

1. S. E. L. A., Mager, L. Breakfield, X. O. & Wood, M. J. Extracellular vesicles: biology and emerging therapeutic opportunities. Nat. Rev. Drug Discov. 12, 347–357 (2013).
2. Hoshino, A. et al. Tumour exosome integrins determine organotropic metastasis. Nature 527, 329–335 (2015).
3. Costa-Silva, B. et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. Nat. Cell Biol. 17, 816–826 (2015).
4. Yoshouka, Y. et al. Ultra-sensitive liquid biopsy of circulating extracellular vesicles using ExoScreen. Nat. Commun. 5, 3991 (2014).
5. Santiago-Dieppa, D. R. et al. Extracellular vesicles as a platform for ‘liquid biopsy’ in glioblastoma patients. Expert Rev. Mol. Diagn. 14, 819–825 (2014).
6. Poljakov, A., Spilman, M., Dokland, T., Amling, C. L. & Mobley, J. A. Structural heterogeneity and protein composition of exosome-like vesicles (prostasomes) in human semen. Prostate 69, 159–167 (2009).
7. Yuana, Y. et al. Cryo-electron microscopy of extracellular vesicles in fresh plasma. J. Extracell. Vesicles https://doi.org/10.34212/j-ev.20.1494 (2013).
8. Tauro, B. J. et al. Two distinct populations of exosomes are released from LIM1863 colon carcinoma cell-derived organoids. Mol. Cell. Proteom. 12, 587–598 (2013).
9. Willms, E. et al. Cells release subpopulations of exosomes with distinct molecular and biological properties. Sci. Rep. 6, 22519 (2016).
10. Larssen, P. et al. Tracing cellular origin of human exosomes using multiplex proximity extension assays. Mol. Cell. Proteom. 16, 1679–1687 (2017).
11. Castillo, J. L. et al. Surfaceome profiling enables isolation of cancer-specific exosomal cargo in liquid biopsies from pancreatic cancer patients. Ann. Oncol. 29, 223–229 (2017).
12. Tavassolidana, G. et al. Multiple recognition assay reveals prostasomes as promising plasma biomarkers for prostate cancer. Proc. Natl Acad. Sci. USA 108, 8809–8814 (2011).
13. Herreros-Villanueva, M. & Bijnard, L. Glypicann-1 in exosomes as biomarker for early detection of pancreatic cancer. Ann. Transl. Med. 4, 64 (2016).
14. Peinado, H. M. et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat. Med. 18, 883–891 (2012).
15. Erdbreugger, U. et al. Imaging flow cytometry elucidates limitations of microparticle analysis by conventional flow cytometry. Cytom. Part A Int. Soc. Anal. Cytol. 85, 756–770 (2014).
16. Im, H. et al. Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. Nat. Biotechnol. 32, 490–495 (2014).
17. Sano, T., Smith, C. L. & Cantor, C. R. Immuno-PCR: very sensitive antigen detection by means of specific antibody-DNA conjugates. Science 258, 120–122 (1992).
18. Fredriksson, S. et al. Protein detection using proximity-dependent DNA ligation assays. Nat. Biotechnol. 20, 473–477 (2002).
19. Soderberg, O. et al. Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat. Methods 3, 995–1000 (2006).
20. Lof, L. et al. Detecting individual extracellular vesicles using a multicolor in situ proximity ligation assay with flow cytometric readout. Sci. Rep. 6, 34358 (2016).
21. Kivioja, T. et al. Counting absolute numbers of molecules using unique molecular identifiers. Nat. Methods 9, 72–74 (2011).
22. Fire, A. & Xu, S. Q. Rolling replication of short DNA circles. Proc. Natl Acad. Sci. USA 92, 4641–4645 (1995).
23. de Gassart, A., Geminard, C., Fevrier, B., Raposo, G. & Vidal, M. Lipid raft-associated protein sorting in exosomes. Blood 102, 4336–4344 (2003).
Acknowledgements
This work was funded by the Swedish Research Council, Ingbrit och Arne Lundbergs Forskningsstiftelse, Knut and Alice Wallenberg Foundation, the European Research Council under the European Union’s Seventh Framework Programme (FP7/2007–2013), ERC grant agreement N° 294409 (ProteinSeq) and Marie Curie ITN grant agreement N° 316929 (GastricGlycoExplorer).

Author contributions
D.W. conceived the proximity barcoding method. D.W. and J.Y. designed and performed the experiments. Q.S., L.W., L.D. and G.R. prepared the exosomes. Y.C. performed the NTA analysis of exosomes and RCPs. Y.S. constructed the bioinformatics pipeline. D.W., J.Y., X.S., M.T., J.O. and X.Q. analyzed the data and interpreted the results. M.N., U.L., and M.K-M. supervised the project. D.W., J.Y., U.L. and M.K-M. wrote and all authors read and commented on the manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-11486-1.

Competing interests: D.W. has filed a patent application (PCT/SE2014/051133) describing the PBA technique. D.W., U.L., J.Y. and M.K-M hold shares in Vesicode AB commercializing the PBA technology. J.O. is an employee of Vesicode AB. The remaining authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Peer review information: Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.