Coated Latex Beads as Artificial Cells for Quantitative Investigations of Receptor/Ligand Interactions

Doris Urlaub¹,² and Carsten Watzl¹,²

¹Department for Immunology, Leibniz Research Centre for Working Environment and Human Factors (IfADo) at TU Dortmund, Dortmund, Germany
²Corresponding authors: urlaub@ifado.de; watzl@ifado.de

Cellular interactions are often essential to regulate immune cell activities during an immune response. To understand the details of this process, it is necessary to study individual receptor/ligand interactions in a quantitative fashion. However, this is often very difficult or even impossible when using real cells for stimulation. Here, we present a method to use cell-sized latex beads for such studies. These beads can be coated with agonistic antibodies or specific ligands in a defined and quantifiable fashion. This creates the possibility of titrating the strength of the stimulation for a specific receptor in a three-dimensional system. Using natural killer (NK) cells as an example, we demonstrate how these beads can be used to stimulate NK cell responses. © 2020 The Authors.

Basic Protocol 1: Covalent coating of latex beads with antibodies
Basic Protocol 2: Quantification of the amount of antibodies on the beads with the QIFIKIT®
Alternate Protocol 1: Covalent coating of latex beads with streptavidin to bind biotinylated proteins
Alternate Protocol 2: Quantification of the amount of protein on the beads with the QIFIKIT®
Support Protocol: Functional testing of the beads in a natural killer cell degranulation assay

Keywords: beads • flow cytometry • quantification • stimulation

INTRODUCTION

In vitro stimulation of immune cells can be carried out using a large variety of methods. Stimulation by cell/cell interaction, e.g., T cells with antigen-presenting cells or natural killer (NK) cells with tumor target cells, mimics the physiological condition most closely. However, using cells to stimulate other cells also entails a number of disadvantages. Even if the cells are properly characterized, there is always the chance that unknown interactions will influence the observed result or that the “stimulating” cell will cause results that will then be attributed to the “stimulated” cell. In addition, it is difficult to study
quantitative aspects of the stimulation as it is not easy to vary and to quantify the amounts of stimulating ligands on cells.

Agonistic antibodies have a clear advantage in that only the intended receptor is triggered and that the stimulation strength can be modified by titration of the antibody (Mesecke, Urlaub, Busch, Eils, & Watzl, 2011). In most cases, crosslinking, e.g., with a secondary antibody, is necessary to induce clustering of the receptor and generate activation signals. This is considered to be artificial, and the effects may not be comparable to the outcome of stimulation under physiological conditions. In addition, the affinity of the interaction and the binding site can be different from those of the true ligand, and the stimulation can happen all over the cell surface and is not localized like in an immunological synapse.

Another method with some of the advantages offered by the use of antibodies is to prepare surfaces: either antibody-coated slides or lipid bilayers containing the proteins of interest. Using these surfaces to stimulate immune cells is an option if the readout is going to be by microscopy or if the supernatant will be analyzed for soluble molecules, but it complicates the assay and can distort the results if the analysis is intended to be done by flow cytometry.

Therefore, we developed a method using latex beads that have approximately the same size as immune cells for the stimulation of NK cells (Dorsch et al., 2020). The interaction of these beads with the cells matches the proportions of an immunological synapse, and they easily can be included in flow cytometry measurements. Here, we describe how to coat these beads (Basic Protocol 1) and quantify the exact amounts of antibodies that are bound on the surface (Basic Protocol 2) and how to perform functional testing with NK cells (Support Protocol).

The same method can also be used to coat recombinant proteins, e.g., the extracellular domains of the natural ligands of NK cell receptors, onto the beads. By first coating the beads with streptavidin (Alternate Protocol 1) and using recombinant proteins with a targeted biotinylation, the correct orientation of the proteins on the beads can be ensured (Alternate Protocol 2).

STRATEGIC PLANNING

Each coating needs to be quantified (Basic Protocol 2 and Alternate Protocol 2) because the results may vary between different experiments. Keep in mind that you should prepare enough beads not only for your functional experiment (Support Protocol) but also for the quantification, staining controls, and counting. Given that the beads are very stable, they can be prepared in bigger batches once the method is established. Then, multiple experiments can be performed using the same batch of beads.

COVALENT COATING OF LATEX BEADS WITH ANTIBODIES

The reaction partners first need to be activated with a chemical crosslinker to allow covalent binding of the antibodies to the beads. The reaction using EDAC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloride] should mainly result in amide bonds between any carboxyl groups of the antibodies and the amine groups of the beads, but EDAC has no specificity for this reaction, so crosslinking of different reactive groups of the antibodies is also possible. The concentration of EDAC, the sequence in which the different reagents are added, and the timing of the incubation, therefore, are critical.

**Materials**

Aliphatic Amine Latex Beads [2% (w/v); ~3 × 10⁷/ml; Thermo Fisher Scientific, SKU no. A37374; currently only available as custom-order product]

1 × MES buffer (pH 4.8) with or without 0.1% (w/v) SDS (see recipe)
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloride (EDAC; Roth, cat. no. 2156)
Anhydrous dimethylsulfoxide (DMSO; Invitrogen, cat. no. D12345)
Unconjugated monoclonal mouse IgG antibody
Bovine serum album (BSA), fraction V (PAN Biotech, cat. no. P06-1391500)
Dulbecco’s phosphate-buffered saline (DPBS; no calcium, no magnesium; Gibco/Thermo Fisher Scientific, cat. no. 14190169)
Storage buffer: PBS with 0.1% (w/v) glycine (AppliChem, cat. no. 131340) and 0.1% (w/v) sodium azide (Roth, cat. no. K305)

Conical centrifuge tube (e.g., 1.5- or 15-ml)
Microcentrifuge or centrifuge with swinging bucket
Cell counter (e.g., CASY) or hemocytometer and microscope
End-over-end rotator

1. Use approximate concentration of the Aliphatic Amine Latex Beads as delivered (3 \times 10^7/ml) to estimate the volume needed for the entire experiment. Mix beads well and place this volume plus \sim10% more (to account for variations and loss during sample processing) in an appropriate conical centrifuge tube (e.g., a 1.5-ml tube for volumes <400 \mu l or a 15-ml tube for larger volumes).

2. Wash beads twice with an excess (at least twice the volume of the bead suspension) of 1× MES buffer with 0.1% SDS. Mix well and spin down 5 min at 5000 \times g in a microcentrifuge or 1000 \times g in a centrifuge with a swinging bucket. Aspirate supernatant carefully so as not to disrupt the pellet.

   Always store the 1× MES buffer with 0.1% SDS at room temperature and check for precipitates of SDS before use. If necessary, warm the buffer to 37°C to completely dissolve the SDS.

   You can minimize loss of beads by using a centrifuge with a swinging bucket. In any case, be careful because the pellet is fragile.

3. Dilute beads in 1× MES buffer with 0.1% SDS, count beads using a cell counter (e.g., CASY) or a hemocytometer and microscope, and wash for a third time (see step 2). Calculate exact amount of beads in the tube and resuspend them in 30 μl of 1× MES buffer with 0.1% SDS per 1 million beads (3.3 \times 10^7/ml).

   Using a CASY cell counter with settings identical to those for lymphocytes works fine, but you can also count with a hemocytometer as the beads are as big as lymphocytes. You should dilute the bead sample appropriately for the counting method that you are using. With our CASY settings, a pre-dilution of 1:10 compared to the initial volume of the bead suspension is usually within the good range.

4. Add EDAC in anhydrous DMSO to beads to a final concentration of 10 mg/ml.

   EDAC hydrolyzes pretty quickly just by opening the container repeatedly and loses its activity. We recommend preparing a 100 mg/ml stock solution in water-free DMSO and storing it at −20°C because it can be reused several times without a reduction in activity. EDAC does not dissolve well in DMSO, so you might have to warm the tube to 37°C and vortex vigorously.

5. Incubate for 30 min at room temperature on an end-over-end rotator.

6. Wash beads once with an excess of 1× MES buffer with 0.1% SDS (see step 2) and resuspend in 30 μl of 1× MES buffer (without SDS) per 1 million beads (3.3 \times 10^7/ml).

7. Add desired amount of unconjugated monoclonal mouse IgG antibody to the beads and incubate for 1 hr at room temperature on an end-over-end rotator.
Use a standardized unit to make comparisons between experiments possible, e.g., \( \mu g \) antibody per million beads. Concentrations between 10 ng and 2 \( \mu g \) per million beads should yield good titrations.

Include at least one sample without antibody and, if applicable, another negative control appropriate to your experiment.

8. Add 20 \( \mu g \) BSA per million beads to bead/antibody mix and continue incubation on rotator for an additional 30 min.

   A stock solution of BSA of 10 \( \mu g/\mu l \) in 1 \( \times \) MES buffer is convenient here.

   Also add this to the sample without antibody.

9. Wash beads twice with an excess of DPBS (see step 2).

   Count the beads diluted in DPBS (see step 3) before the final wash step because some beads will be lost during the coating process and this may vary between samples. To make sure that the correct amount is used in your experiments and that different samples are comparable, it is essential to adjust concentrations.

10. Use beads directly or resuspend beads in storage buffer, e.g., at a concentration of 1 \( \times \) \( 10^7 \) beads/ml, and store at 4°C until use (see Basic Protocol 2).

   See the Commentary for information about the stability of the coated beads.

**BASIC PROTOCOL 2**

QUANTIFICATION OF THE AMOUNT OF ANTIBODIES ON THE BEADS WITH THE QIFIKIT®

The manual for the QIFIKIT® provides plenty of information on how different samples can be prepared and analyzed. Nevertheless, we describe here how we adapted this protocol to fit the requirements of our assay.

**Materials**

- QIFIKIT®, including tubes containing setup and calibration beads and labeled secondary antibody (Dako/Agilent, cat. no. K0078)
- Antibody-coated latex beads (see Basic Protocol 1)
- FACS buffer [PBS with 2% (v/v) fetal bovine serum (FBS)] with or without 2% (w/v) formaldehyde
- 96-V-well plate
- Centrifuge with swinging bucket
- Flow cytometer and analysis software
- Spreadsheet program

1. Vortex tubes containing the setup beads and the calibration beads of the QIFIKIT® and transfer 50 \( \mu l \) of each to a 96-V-well plate.

   The QIFIKIT® manual uses 100 \( \mu l \) each, but results using fewer beads are comparable.

   You can also perform the staining in other vessels, but using 96-V-well plates worked best in our hands to reduce sample loss and to guarantee equal treatment and comparability of samples.

2. Take same amount of antibody-coated latex beads to be quantified from each condition (100,000 to 200,000 beads per sample) and transfer these samples to 96-V-well plate. Include one sample as an unstained control.

3. Wash QIFIKIT® beads and latex beads once by adding 150 \( \mu l \) FACS buffer to each well of the 96-V-well plate, centrifuging 5 min at 500 \( \times \) g in a centrifuge with a swinging bucket, and aspirating the supernatant.
4. Prepare a master mix of labeled secondary antibody in FACS buffer. Resuspend all beads except unstained control (just QIFIKIT® beads and latex beads) in 50 μl of this master mix. Incubate for 20 min at room temperature in the dark.

The QIFIKIT® contains a FITC-labeled anti-mouse antibody, which should be diluted 1:50 and works fine. However, we usually use a PE-labeled goat anti-mouse antibody (e.g., Goat Anti Mouse F(ab)_2, IgG (H+L) PE, Dianova, cat. no. 115-116-146) diluted 1:200 because we found that the assay was more sensitive, with a better, lower detection limit, when we used the brighter PE fluorophore.

5. Wash once with 150 μl FACS buffer (see step 3) and resuspend beads in 100 to 150 μl FACS buffer with or without 2% (w/v) formaldehyde per sample.

Use FACS buffer if you can analyze the samples directly or FACS buffer with 2% formaldehyde to fix the samples and analyze the beads the next day.

Make sure the QIFIKIT® beads are treated in the same way as the other samples.

6. Analyze samples using a flow cytometer.

The beads from the QIFIKIT® and the latex beads have very similar FSC and SSC signals. Adjust the amplification so that you can nicely separate beads from debris and draw a gate in FSC and SSC around the main population, excluding debris and aggregates (Fig. 1A and 1D). Look at events from this gate in the fluorescence channel that you used (FITC or PE) on a logarithmic scale. Set the amplification of this channel so that you can see both populations of the setup beads (Fig. 1B). The higher population may be close to, but not at, the maximal detection limit. Then, record the setup and calibration beads and your latex beads without changing the settings.

7. Perform analysis using analysis software and a spreadsheet program.

In the analysis software, you now need to gate on single beads using FSC and SSC signals. Display the fluorescence channel as a histogram. For the beads from the QIFIKIT®, you need to draw additional gates around the individual populations: two in the setup beads and five in the calibration beads (Fig. 1B and 1C). Then, export the mean fluorescence intensity (MFI) values of these populations to a table or spreadsheet program.

If the latex beads are coated homogeneously, you will see only one narrow peak, but, especially at very low antibody concentrations, the coating can be heterogeneous. In the case that you have more than one peak or a very smeared population, we recommend repeating the coating (see Basic Protocol 1).

For the calibration beads, the QIFIKIT® provides information about the exact number of antibodies bound to the surface, which is called antibody binding capacity (ABC), for each of the five populations. Using the logarithm of the ABC and the logarithm of the corresponding MFI, a linear regression can be calculated:

\[ \log(ABC) = a \times \log(MFI) + b \]

Using this formula, the ABC of the latex beads can be calculated from their MFI. Also perform this calculation for the negative-control beads without antibody coating that have been stained in the same way. Subtract this from the ABCs of the other beads for background correction.

COVALENT COATING OF LATEX BEADS WITH STREPTAVIDIN TO BIND BIOTINYLATED PROTEINS

Following the steps of Basic Protocol 1, virtually any protein can be covalently bound to the beads; we use this protocol to coat the beads with streptavidin at one constant concentration. These beads can then bind any biotinylated protein. The amount of protein on the surface can be modified by titrating the recombinant biotinylated protein. Additionally, when the biotinylation of the protein is targeted, correct orientation on the beads can be ensured.
Figure 1  Analysis of the antibody coating with the QIFIKIT®, with exemplary quantification of antibody-coated beads. (A) Beads from the QIFIKIT® are first gated via their FSC and SSC signals. A gate is drawn around the main population of single beads and excludes debris and aggregates. (B) The fluorescence channel is set to logarithmic scale, and both populations of the setup beads must be visible. (C) The five populations of the calibration beads are clearly separated. All beads have to be recorded without changing the settings of the fluorescence channel. (D) The latex beads have very similar FSC and SSC signals as the beads from the QIFIKIT®, so the same settings and gating can also be applied here. (E) The beads coated with αNKp30 show only one narrow peak at all antibody concentrations and therefore are homogeneously coated. (F) Two populations among the beads coated with αNKG2D show heterogeneous coating: at 80 ng/Mio, there are two peaks visible, and at 250 ng/Mio, the population is smeared across a wide range. Depending on the intended use of the beads, those samples might have to be excluded. (G) After drawing gates around the individual populations of the setup and the calibration beads (B and C), a linear regression is calculated from the mean fluorescence intensity (MFI) and the antibody binding capacity (ABC) values of the five calibration bead populations using a spreadsheet program. With the linear regression, the ABCs of the beads coated with αNKp30 can be calculated from their MFI values (G, gray symbols).

Additional Materials (also see Basic Protocol 1)

Streptavidin (BioLegend, cat. no. 405150)
FACS buffer: PBS with 2% (v/v) FBS
Biotinylated protein (biotin: Sigma, cat. no. B4501)

1. Follow steps 1 to 6 of Basic Protocol 1.
2. Add 0.5 μg streptavidin per million beads and incubate for 1 hr at room temperature on an end-over-end rotator.

   Here, we do not use uncoated beads as a negative control, but rather streptavidin beads without biotinylated protein added (see step 4).

3. Continue with steps 8 and 9 of Basic Protocol 1.

4. Resuspend beads in storage buffer, e.g., at a concentration of $1 \times 10^7$ beads/ml, and store them at 4°C. To bind biotinylated protein to the beads, incubate beads for 20 min at room temperature in FACS buffer containing the biotinylated protein.

   Similar to direct coating with antibodies (see Basic Protocol 1), concentrations between 10 ng and 2 μg per million beads have yielded good titrations in our experiments. Include a negative control without protein.

   Free streptavidin on the beads might interact with biotin in later stages of your experiment if the sites are not already blocked, e.g., by incubation in cell culture medium that contains biotin. In the case that you expect that free streptavidin could cause problems, add an additional blocking step with 10 μM biotin for 20 min at room temperature.

5. Wash beads twice with an excess of FACS buffer (see step 2 of Basic Protocol 1).

6. Use beads directly or keep them at 4°C in storage buffer.

   See the Commentary for information about the stability of the coated beads.

### QUANTIFICATION OF THE AMOUNT OF PROTEIN ON THE BEADS WITH THE QIFIKIT®

To quantify the protein loaded onto the beads with Alternate Protocol 1, one additional step is needed relative to Basic Protocol 2, but it can be done very easily with the QIFIKIT®.

**Additional Materials (also see Basic Protocol 2)**

- Streptavidin-coated latex beads with or without biotinylated protein (see Alternate Protocol 1)
- Unconjugated monoclonal mouse IgG antibodies against biotinylated proteins used (see Alternate Protocol 1)

1. Take same amount of streptavidin-coated latex beads with biotinylated protein to be quantified from each condition (100,000 to 200,000 beads per sample) and transfer them to a 96-V-well plate. Include one sample of streptavidin-coated latex beads without biotinylated protein for each antibody that is going to be used. If the beads are in storage buffer, wash them once by adding 150 μl FACS buffer to each well of beads in the 96-V-well plate, centrifuging 5 min at 500 $\times$ g, and aspirating supernatant.

2. Resuspend beads in 50 μl FACS buffer containing 5 μg/ml unconjugated monoclonal mouse IgG antibody against the respective biotinylated protein used. Treat sample of streptavidin-only beads in the same way. Incubate for 20 min at room temperature.

   The antibody concentration must be saturating so that one epitope on the bead corresponds to one antibody bound.

3. Wash beads once with 150 μl FACS buffer. Prepare setup and calibration beads from the QIFIKIT® as in Basic Protocol 2, steps 1 and 3.

4. Prepare a master mix of labeled secondary antibody in FACS buffer and resuspend all samples in 50 μl of this master mix. Incubate for 20 min at room temperature in the dark.

   See the annotation to Basic Protocol 2, step 4, for more information about the antibody.
5. Follow steps 5 to 7 of Basic Protocol 2.

The ABC of the latex beads can be calculated in the same way as in Basic Protocol 2. Use the beads coated with streptavidin only and stained with the same antibody for background correction and subtract this from the ABC of the protein-coated beads.

**FUNCTIONAL TESTING OF THE BEADS IN A NATURAL KILLER CELL DEGRANULATION ASSAY**

One of the main functional assays that we use and an excellent way to test beads that are coated with antibodies (Basic Protocol 1) that are directed against activating NK cell receptors is a degranulation assay that detects surface CD107a. We use antibodies to coat the beads that have been successfully used in our laboratory to stimulate NK cells via other methods.

**Additional Materials** *(also see Basic Protocol 2)*

- Preferred culture medium for NK cells
- NK cells (e.g., primary human NK cells or NK-92 cell line)
- αCD107a (clone H4A3) PE-Cy5 (BD Pharmingen, cat. no. 555802)

*NOTE:* All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

*NOTE:* All culture incubations are performed in a humidified 37°C, 5% CO₂ incubator unless otherwise specified.

1. Take same amount of antibody-coated latex beads from each sample to test (e.g., 1 × 10⁵) and transfer them to a 96-V-well plate.

2. Wash beads once with 100 μl of the preferred culture medium for NK cells to remove the storage buffer. Centrifuge 5 min at 500 × g in a centrifuge with a swinging bucket and aspirate supernatant.

3. Count NK cells, centrifuge 5 min at 500 × g if necessary, and resuspend the same number of cells as beads (1:1 ratio) at a concentration of 2 × 10⁶ cells/ml in culture medium.

   *As beads and NK cells are used at a 1:1 ratio, for example, you need 1 × 10⁵ NK cells per sample of 1 × 10⁵ beads and 2 × 10⁵ NK cells for control samples.*

4. Allocate NK cells (e.g., 1 × 10⁵ NK cells in 50 μl) as an unstained control in an empty well of the 96-V-well plate. Add 0.2 μg/ml αCD107a PE-Cy5 to remaining NK cell suspension and mix by carefully inverting tube. Remove NK cells (e.g., 1 × 10⁵ NK cells in 50 μl) as an unstimulated control. Then, resuspend beads in 50 μl NK cell mix per sample.

5. Incubate 96-V-well plate containing control cells and samples mixed with beads for 3 hr in a humidified incubator at 37°C and 5% CO₂.

6. Wash all samples once with 150 μl FACS buffer and resuspend them in 150 μl FACS buffer.

7. Analyze samples directly using a flow cytometer or store them for a short time at 4°C protected from light before flow cytometry.

8. Perform analysis using analysis software and a spreadsheet program.

*Although the latex beads are similar in size to NK cells, the SSC signals are very different. Adjust the amplification of both scatters so that both the NK cell and the latex bead populations are visible. Draw gates around the NK cell population, excluding cell debris, and*
Degranulation assay with primary NK cells and latex beads. The amplification of the SSC must be adjusted so that both the NK cell and the latex bead populations are visible (A). Beads without antibody do not form many conjugates with the NK cells, but the gating should include possible conjugates, as with αCD16-coated beads (C). With the bead autofluorescence that causes a higher signal at 450 nm after excitation at 405 nm, single NK cells can be distinguished from beads as well (B and D). The gates for αCD107a-positive events are set very strictly to only include the highly positive events. This is to avoid beads that had contact with a degranulating cell and that also show slight staining with αCD107a (see D, Q1) being accidentally counted as NK cells.

Figure 2  Degranulation assay with primary NK cells and latex beads. The amplification of the SSC must be adjusted so that both the NK cell and the latex bead populations are visible (A). Beads without antibody do not form many conjugates with the NK cells, but the gating should include possible conjugates, as with αCD16-coated beads (C). With the bead autofluorescence that causes a higher signal at 450 nm after excitation at 405 nm, single NK cells can be distinguished from beads as well (B and D). The gates for αCD107a-positive events are set very strictly to only include the highly positive events. This is to avoid beads that had contact with a degranulating cell and that also show slight staining with αCD107a (see D, Q1) being accidentally counted as NK cells.

To analyze the percentage of degranulating NK cells, you can combine the NK cell gate and the latex bead gate in a boolean OR gate. Look at events within this combined gate using the bead autofluorescence and the PE-Cy5 signal (Fig. 2B and 2D). In Quadrant 1 (Q1), you see single beads. Q4 is NK cells that have not degranulated, Q3 is NK cells that have degranulated, and Q2 is NK cells that have degranulated and are still attached to a bead. The formula to calculate the percentage of degranulating NK cells is

\[
\frac{(Q2 + Q3)}{(Q2 + Q3 + Q4)} \times 100.
\]

Of course, other gating strategies can also be applied; just make sure to exclude the beads and not to “lose” the conjugates when you want to analyze NK cells.
REAGENTS AND SOLUTIONS

**MES buffer (pH 4.8), 1x**

To prepare a 10× stock solution of MES, dissolve MES [2-(N-morpholino)ethanesulfonic acid sodium salt, Calbiochem, cat. no. 475894] in ultrapure water to 0.25 M, adjust pH to 4.8 with 1 M HCl, and filter through a 0.22-μm filter. Store sterile 10× stock for ≤1 year at room temperature. Dilute to 1× (0.025 M MES) in ultrapure water and check pH again. Store ≤1 year at room temperature.

**MES buffer (pH 4.8), 1x, with 0.1% SDS**

Prepare a 5% (w/v) SDS stock solution from sodium dodecyl sulfate (SDS; grained pure; AppliChem, cat. no. A7249) in ultrapure water. Warm solution to 37°C if necessary and stir well until SDS is completely dissolved. Store SDS stock for ≤1 year at room temperature. Add this SDS stock 1:50 to 1× MES buffer, pH 4.8 (see recipe). Store ≤2 months at room temperature.

**COMMENTARY**

**Background Information**

Various beads have been used to stimulate NK cells or other immune cells. Some commercial kits use beads to expand cells in culture (e.g., Miltenyi B Cell Expansion Kit). Magnetic beads that are manufactured to isolate cells or proteins can be diverted from this use and coated with antibodies or proteins to stimulate cells (Sim et al., 2019, Todros-Dawda, Kveberg, Vaage, & Inngjerdingen, 2014). However, none of these methods has all properties in the combination that we aim for in our experiments: we want beads that have the same size as cells to mimic a real immunological synapse, that have covalent coating for stability, that support the possibility of titrating and quantifying the coating, and that have an autofluorescence comparable to that of cells to facilitate flow cytometric analysis.

**Critical Parameters**

**Detergent use and centrifugation of the latex beads**

The latex beads are delivered as a surfactant-free suspension. We tested various detergents to find one that facilitates bead handling without negative effects on the coating efficiency. However, even with the addition of SDS to the MES buffer, the beads are difficult to handle during all steps before they are coated with protein (Basic Protocol 1 and Alternate Protocol 1). The centrifugation steps should be performed in a centrifuge with a swinging bucket to minimize loss of beads during the washing steps or must be performed at higher speed. The settling speed of the beads is not the limiting factor, but rather their tendency to stick to all surfaces and to not form a proper pellet. Once the bead surface is coated with protein, slower centrifugation speeds are sufficient, but we still recommend using a centrifuge with a swinging bucket.

**EDAC stability**

EDAC (Basic Protocol 1 and Alternate Protocol 1) hydrolyzes and loses its activity in the presence of even small amounts of humidity. Solutions in aqueous media have to be prepared freshly for each experiment. We observed that even when condensation was avoided, EDAC lost its activity after the container had been opened several times. Therefore, we make “aliquots” of ~10 mg and store them dry at −20°C with desiccant. From these aliquots, you can prepare a fresh solution in MES buffer for each experiment. Because small amounts of EDAC are required frequently, especially while establishing the method, preparing a stock solution in water-free DMSO is the most practical option. EDAC does not dissolve well in DMSO, but a concentration of 100 mg/ml is possible, and this solution can be stored at −20°C and reused several times without a reduction in activity.

**Bead stability**

In the storage buffer, which contains sodium azide as a biocide, the beads (Basic Protocol 1 and Alternate Protocol 1) can be stored for ≥2 months at 4°C without loss of activity. In our initial experiments, we quantified the beads a second time after 8 or 9 weeks and found no substantial changes: neither the ABC nor the functional activity was reduced. Even after 2 years, the ABC of antibody-coated beads (Basic Protocol 1) was within the same range as directly after the coating reaction.
Troubleshooting

**Buffer conditions for bead coating**

The buffer used before and during coating (Basic Protocol 1 and Alternate Protocol 1) can influence the efficiency drastically. MES buffer with pH 6 is recommended by the bead manufacturer, but different proteins might require different pH conditions. The results for coating antibodies were most stable when we used MES buffer with pH 4.8; therefore, we use this pH throughout our experiments. If you have to optimize the protocol because the coating is not sufficient, changing the pH of the MES buffer might work.

As mentioned above, a detergent in the buffer facilitates handling of the beads during the initial washing steps. We tested various detergents and found some that did not disturb the coating process and with which the efficiency was comparable to the coating without detergent. SDS even increased antibody binding to the beads when used before coating, but during the incubation of beads with the antibodies, MES without detergent should be used. If the coating does not work properly, reducing the SDS concentration or using other detergents like Triton-X-100 or Igepal might help, but we have not tested beads coated this way as thoroughly as the ones created with the protocols described (Basic Protocol 1 and Alternate Protocol 1).

**Concentration of EDAC and timing of EDAC addition**

The concentration of EDAC used and when it is applied are also critical (Basic Protocol 1 and Alternate Protocol 1). We thought that co-incubating EDAC together with beads and antibody should lead to a more efficient coating of the beads, but this did not work for various EDAC concentrations tested. The beads generated this way indeed had a high density of antibodies on the surface, but these antibodies were not functional. We suspect that reactions between reactive groups of the antibodies destroyed their structure, including the antigen-binding site. We found that pre-incubation of the beads with a quite high EDAC concentration and washing it out before the antibodies are added leads to the best results. Thus, if you observe high coating of the beads but without the expected functional effects of the antibody, reducing the EDAC concentration or intensifying the washing after the EDAC incubation could be the solution.

**Heterogeneous coating**

Especially at lower antibody concentrations, we often observe heterogeneous coating of the beads (Basic Protocol 1 and Alternate Protocol 1). To reduce this, mixing of samples throughout the incubations with EDAC and with antibodies is essential. To ensure equal distribution of the antibody, we prepare the dilution in MES buffer. After the incubation with EDAC, the beads are washed and resuspended directly in the antibody dilution.

**Some antibodies just do not work**

Maybe due to their individual amino acid sequences or due to the positions of the reactive groups in the structure of the antibodies, some antibody clones just do not bind efficiently to the beads irrespectively of all coating conditions (Basic Protocol 1 and Alternate Protocol 1). Among the different antibodies that we use, the maximal coating differs a lot and is specific to the individual antibody. As long as the coating density is sufficient to induce the functional effects in NK cells (Support Protocol), we still can use those beads in our experiments. In contrast, some antibodies have such a low coating density that we cannot use them to stimulate our cells and have to exclude them from our experiments.

**Understanding Results**

The quantification with the QIFIKIT® (Basic Protocol 2 and Alternate Protocol 2) gives you the total number of antibodies or proteins on one bead. However, depending on the cell type that you use and the cells’ activation state, cells may only make contact with a part of the bead surface. You could estimate the average contact area for your cells by microscopic analysis. Therefore, the number of antibodies that stimulate a certain effect will be lower than the total number of antibodies on a bead.

**Time Considerations**

The coating of the beads (Basic Protocol 1 and Alternate Protocol 1) typically requires <2.5 hr of total incubation time. Depending on how many different conditions you plan to prepare, it can be done within 3 hr. The staining for the quantification of antibody-coated beads and beads coated with biotinylated protein (Basic Protocol 2 and Alternate Protocol 2) can be done in 1 to 1.5 hr. The time that you need for measuring the samples and analyzing the result (Basic Protocol 2 and Alternate Protocol 2) then depends mainly on the number of samples that you prepared. Because the beads can be stored after coating, you do not need to perform these experiments the same day.
The functional assays (Support Protocol) that you want to perform with those beads also can be planned independently once you have prepared the beads. A small degranulation assay with fewer than 10 samples requires <30 min preparation time and can be completed within 5 hr, including the incubation time, measurement, and analysis.

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Author Contributions
Doris Urlaub Investigation; methodology; writing-original draft. Carsten Watzl Conceptualization; funding acquisition; project administration; supervision; writing-review & editing.

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