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Use of Nanobarcodes® Particles in Bioassays

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Summary

We have developed striped metal nanoparticles, Nanobarcodes® particles, which can act as encoded substrates in multiplexed assays. These particles are metallic, encodeable, machine-readable, durable, submicron-sized tags. The power of this technology is that the particles are intrinsically encoded by virtue of the difference in reflectivity of adjacent metal stripes. This chapter describes protocols for the attachment of biological molecules, and the subsequent use of the Nanobarcodes particles in bioassays.

Key Words

Multiplex; substrate; nanoparticles; hybridization; immunoassay; fluorescence microscopy.

1. Introduction

1.1. Needs of the Bioassay Community

The need to measure simultaneously many different molecular species in microliters of sample ***(1)*** has propelled bioassays toward multiplexation, miniaturization, and ultrasensitivity. Multiplexing affords the ability to make two or more measurements simultaneously. This has a number of advantages. It reduces the time and cost to collect the measurement. It can also often reduce the amount of sample needed to acquire the measurement. More important, it allows data to be reliably compared across multiple experiments. Additionally, multiplexing can add confidence to measurements through the incorporation of multiple internal controls. Techniques for multiplexing bioassays can be divided into two broad categories: the use of multiple quantitation tags such as

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organic fluorophores, and the use of substrates to achieve spatial multiplexing. Our discussion here concentrates on the latter approach.

A widely used approach to spatial multiplexing is the use of a planar microarray in which the identity of the target analyte is encoded by its location, with a secondary reporter providing the quantitative data (such as an organic fluorescent dye). Many technologies make use of this approach, including oligonucleotide and cDNA microarrays *(2)* and protein arrays *(3)*. An alternative to planar microarrays, which we advocate, is to use solution arrays, in which encoded nanoparticles are used to provide multiplexed data. Compared to microarray-based methods, these "suspension arrays" offer, in principle, greater flexibility (via the ability to incorporate easily a new assay via addition of a new type of bead), more rapid assay times (radial vs planar diffusion), and greater reproducibility (tens to hundreds of thousands of replicates for each assay), and they can potentially use less sample and reagent. Analyte quantitation is achieved by the use of a quantitation tag (typically an organic fluorophore).

1.2. Nanobarcodes® Particles as Encoded Nanoparticles

At Nanoplex Technologies, we have developed striped metal nanoparticles (Nanobarcodes particles) (NBCs) as encoded substrates for multiplexed assays and have previously described their use in multiplexed assays *(4–7)*. NBCs are encodeable, machine-readable, durable, submicron-sized tags. The power of this technology is that the particles are intrinsically encoded by virtue of the difference in reflectivity of adjacent metal stripes, as shown in **Fig. 1**. The large number of striping patterns available and the development of automated software for particle identification should facilitate development of very highly multiplexed assays (i.e., hundreds to thousands). The NBCs are manufactured in a semiautomated, highly scalable process by electroplating inert metals—such as gold (Au), nickel (Ni), platinum (Pt), or silver (Ag)—into templates that define the particle diameter, and then releasing the resulting striped nanorods from the templates *(8)*. Just as a conventional barcode is read by measuring the differential contrast between adjacent black and white lines using an optical scanner, individual NBCs are read by measuring the differential reflectivity between adjacent metal stripes within a single particle using a conventional optical microscope. The advantage of this method of encoding is its massively multiplexed capabilities (e.g., nine stripes of three metals = approx 10,000 combinations). We foresee many applications for NBCs as substrates. One potential application for this technology is the detection of unique DNA sequences for diagnostic applications, including the detection of single-nucleotide polymorphisms (SNPs).

As is the case for other bioassay substrates, such as beads and microarray slides, the active surface of the NBCs must be modified such that the particles

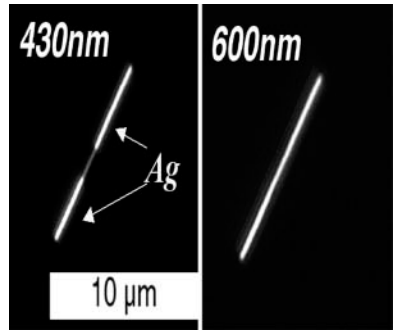

Fig. 1. Differences in reflectivity at 430 vs 600 nm for NBC sequence Ag-Au-Ag.

will bind to target biological molecules. We present here protocols for the attachment of oligonucleotides to the surface of the NBC via amine linkage, the subsequent hybridization of attached oligonucleotides, the derivatization with a universal attachment chemistry, and the subsequent quality control. In addition, we discuss methods of enumeration of particles, detection, and analysis.

2. Materials

1. Benchtop sonicator.
2. Microcentrifuge (set to 5000 rpm).
3. Analytical balance.
4. Pipetmen (P10, P100, and P1000).
5. Hemacytometer and cover slip.
6. Upright optical microscope (with ×20 objective).
7. Rotator.
8. Microcentrifuge tubes (1.5 mL).
9. NBCs in H_2O (Nanoplex Technologies, Menlo Park, CA).
10. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Pierce Biotechnology, Rockford, IL).
11. *N*-Hydroxyl sulfosuccinimide (NHS) (Pierce).
12. NeutrAvidin™ (Molecular Probes, Eugene, OR).
13. Phosphate-buffered saline (PBS), pH 7.4 (Sigma, St. Louis, MO).
14. 2(*N*-Morpholino)ethane sulfonic acid (MES), pH 5.0 (Sigma).
15. Sodium dodecyl sulfate (SDS) (Sigma).
16. 70% Ethanol (Sigma).
17. 20X saline sodium citrate (SSC) (Promega, Madison, WI).
18. PEG-8000 (Promega).
19. Formamide (Sigma).
20. Bovine serum albumin (BSA) (Sigma).
21. Molecular biology-grade distilled water (Promega).

22. EDC/NHS coupling solution: 100 n*M* EDC/40 m*M* NHS in MES buffer (pH 5.0).
23. Hybridization buffer: 6X SSC, 5% PEG-800, 40% formamide.

3. Methods

NBCs, as obtained from Nanoplex Technologies, have a self-assembled monolayer surface coating, resulting in a functionalized surface of carboxyl groups. This allows the use of carbodiimide chemistry to attach primary amine groups of the biomolecule to the carboxyl groups on the NBCs. Carbodiimides couple carboxyl groups to primary amines, resulting in the formation of amide bonds. NHS is included in the reaction in order to stabilize the *O*-acylisourea intermediate product ***(9)***.

Note that in all protocols, particles with different striping patterns are derivatized separately until the final multiplexed assay step. NBCs are supplied in ethanol, and prior to any experiment they must be exchanged into water.

3.1. Attachment of Amine-Derivatized Oligonucleotides to NBCs

Oligonucleotides can be directly attached to the surface carboxyl groups if they are modified with amine groups on the 3′ or 5′ end.

1. Centrifuge 100 µL of NBCs (5×10^6/µL) for 1 min.
2. Incubate the NBCs with 500 µL of EDC/NHS solution for 30 min in 1.5-mL Eppendorf tubes. Use a rotator to mix the reaction continuously during incubation. Alternatively, the particles can be occasionally sonicated to ensure even activation of the particle surface. It is critical that this reaction only proceed for 30 min (*see* **Note 1**).
3. Wash the NBCs with 10 m*M* PBS twice, and resuspend in 100 µL of 10 m*M* PBS.
4. Incubate the activated NBCs with 980 µL of 10 m*M* PBS and 20 µL of 10 µ*M* amine-modified oligonucleotide for 30 min at room temperature on a rotator.
5. Wash the NBCs with 10 m*M* PBS twice. Resuspend the NBCs in 100 µL of 10 m*M* PBS. NBCs derivatized with oligonucleotides can be stored for future use for approx 2 wk at 4°C.

3.2. Hybridization of Oligonucleotides Conjugated to NBCs

Following attachment of oligonucleotides to NBCs, assays often involve a hybridization step. Such an assay can be used to detect SNPs or mutations, or to measure gene expression. The following is a generic protocol for hybridization of oligonucleotides attached to NBCs.

1. To 90 µL of hybridization buffer, add 5 µL of oligonucleotide conjugated to NBCs (between 2.5 and 5×10^7 NBCs) and 5 µL of target DNA (1–10 pmol). Mix well.
2. Incubate at 42°C for 2 h while tumbling on a rotator.

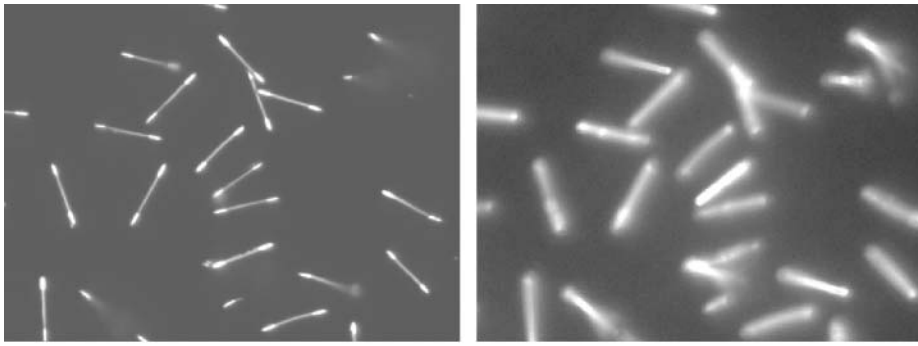

Fig. 2. Signal detection of oligonucleotide-derivatized NBCs hybridized with complementary Cy5 oligonucleotide.

3. Wash the particles with 500 µL of 1X SSC for 5 min at room temperature. Wash the particles with 500 µL of 0.1 X SSC for 5 min at room temperature. Resuspend the particles in 50 µL of 10 m*M* PBS.
4. Image with a fluorescence microscope. (For more details on imaging, *see* **Subheading 3.6.**).

Figure 2 shows a pair of optical microscope images from the protocols in **Subheadings 3.1.** and **3.2.** The left-hand image is a typical reflectance image (405 nm) for a set of particles with the striping pattern 100001. The right-hand image shows the fluorescence from the same particle (620-nm excitation, 690-nm emission) after hybridization with fluorescently labeled oligonucleotide.

3.3. NeutrAvidin Surface Coating of NBCs

A more generic derivation chemistry is to attach a modified avidin to the NBCs, allowing the attachment of any biotin-terminated species, including oligonucleotides for DNA-based assays and biotinylated antibodies for immunoassays. We choose to work with NeutrAvidin because it binds optimally to biotin at neutral pH, making it easier to work with than streptavidin, in which binding is outside physiological pH ranges. Attachment of the NeutrAvidin uses the same chemical reaction as attachment of amine-labeled oligonucleotides, the carbodiimide chemistry.

1. Follow **steps 1–3** in **Subheading 3.1.**
2. After incubation, sonicate the reaction tube to suspend the particles that have adhered to the wall and lid. Add SDS to a final concentration of 0.1% and centrifuge.

3. Wash the particles with 500 µL of 10 m*M* PBS/0.1% SDS twice. Resuspend the particles in 100 µL of 10 m*M* PBS.
4. Prepare 1 mL of 0.1 mg/mL NeutrAvidin in PBS buffer. Mix the activated particles with 800 µL of 10 m*M* PBS and 100 µL of 1 mg/mL NeutrAvidin. *Note:* Always add the particles *to* protein. Briefly sonicate, and allow the mixture to incubate for 30 min on a rotator.
5. After 30 min, sonicate the reaction vessel to suspend the particles adhering to the walls and lid. Add SDS to a final concentration of 0.1% and centrifuge.
6. Suspend the particles in enzyme-linked immunosorbent assay blocking buffer (PBS, 0.5% BSA, 0.1% sodium azide). Note that BSA should not be used at any stage prior to this step, because it will compete with the NeutrAvidin in the attachment step.
7. Wash the NBCs with 10 m*M* PBS twice. Resuspend the NBCs in 100 µL of 10 m*M* PBS. NBCs derivatized with protein can be stored for future use for approx 2 wk at 4°C.

3.4. Quality Control of NeutrAvidin Attachment Step

Quality control can be carried out using a number of approaches. The most informative is to react with fluorescently labeled biotin. However, the protocol described here can also be used to attach another biomolecule of interest, such as a biotinylated protein, an antibody, or an oligonucleotide.

1. To 1×10^6 of NBCs (10 µL NBCs at 1×10^5/µL) add 10 µL of 0.4 µ*M* dye-labeled biotin.
2. Let the reaction proceed for 20 min at room temperature. Be sure to cover the reaction chamber with aluminum foil, to eliminate photobleaching of the fluorescent dye.
3. After 20 min, add 500 µL of PBS/0.5% BSA/0.1% sodium azide with 0.1% SDS. Centrifuge, aspirate the supernatant, and wash three times.
4. Resuspend the NBCs in 100 µL of PBS/0.5% BSA/0.1% sodium azide for imaging.

3.5. Measuring Nanoparticle Concentrations (Particle Enumeration)

During both the methods development process and subsequently when performing quantitative assays, it is important to know the number of NBCs in each assay, to account for variations in particle concentration caused by manufacturing and particle derivatization procedures. We have developed a simple method for enumeration that is applicable for all nanoscale particles and uses a hemacytometer. A standard hemacytometer, normally used to count blood cells, is about the size of a regular microscope slide consisting of two chambers. The chambers are etched glass with raised sides that support a quartz cover slip. The cover slip is exactly 0.1 mm above the chamber floor. The counting chamber is etched in a total surface area of 9 mm^2. Calculation of concentration is based on the volume underneath the cover slip. In our case,

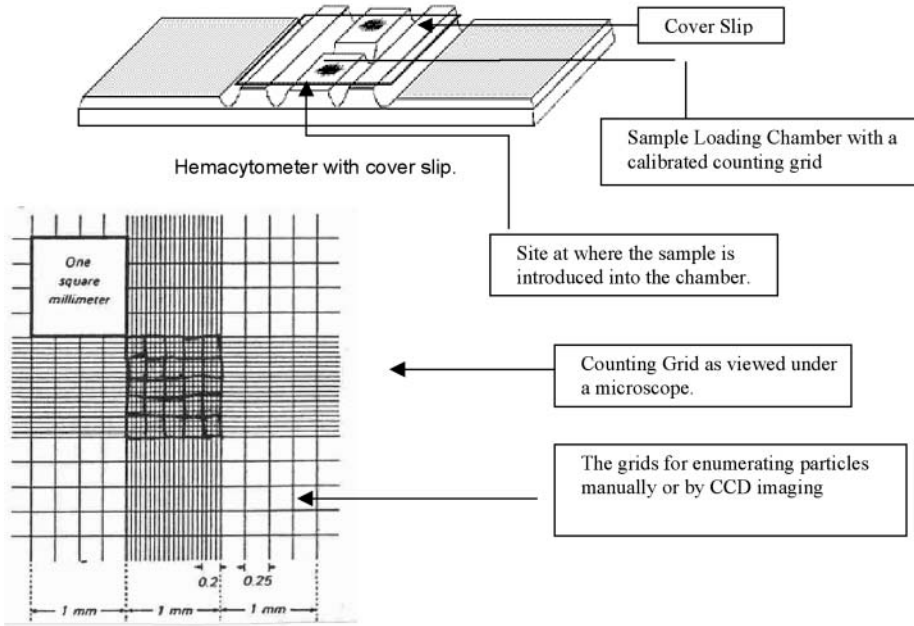

Fig. 3. Layout of hemacytometer.

one large square has a volume of 0.0001 mL (length × width × height; i.e., 0.1 × 0.1 × 0.01 cm). Each large square is divided into nine 1.0-mm squares. The NBC concentration per milliliter will be the average count per square × 10^4. **Figure 3** shows the device as layout, and **Fig. 4** a typical image.

1. Sonicate NBCs to suspend the particles that have adhered to the wall and lid.
2. Make 1 : 10, 1 : 100, and 1 : 1000 serial dilutions of the particles in water.
3. Using a pipetman, load the 1 : 100 dilution into both chambers of the hemacytometer by carefully touching the edge of the cover slip with the pipet tip and allowing the chamber to fill by capillary action. About 10 µL should be enough to load each chamber.
4. Let the particles sediment in the chamber for 5–10 min.
5. Using a ×20 objective, scan each square of the 4 × 4 squares grid (1 mm^2). Check for an even distribution of the particles.
6. Count each 1 or 2 or 3 squares such that a count of 100 particles is obtained. If a total count of 100 particles is not obtained from all 16 squares, repeat the process with the 1 : 10 dilution. If the particle number is much greater than 100 in a single square, repeat with the 1 : 1000 dilution.
7. Calculate the NBC concentration based on the dilution factor and hemacytometer dimensions.

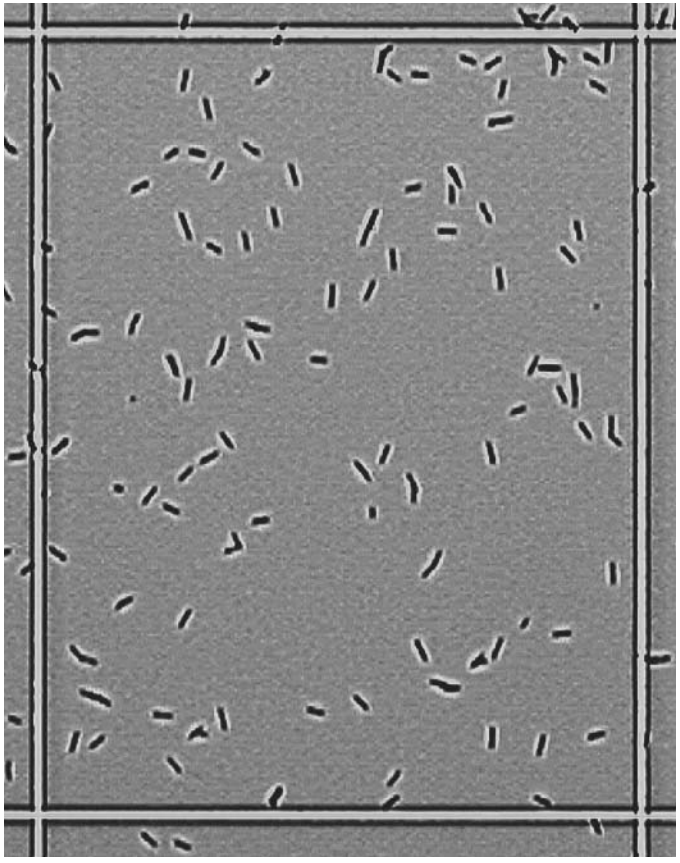

Fig. 4. NBC particles as they appear in 1 of the 16 grids (4 × 4 squares grid) using a ×20 objective lens in a hemacytometer.

3.6. Detection of NBCs

The measurement of NBCs requires the use of a reflectance microscope. If the NBCs are going to be used as substrates in bioassays, the microscope must also be capable of fluorescence measurements. The microscope can be either upright or inverted. The typical fluorescence assay image acquisition process is as follows:

1. Choose the reflectance filter set and focus on the reflectance image of the particles.
2. Acquire and save the reflectance image. Camera integration time is from 10 to 100 ms, depending on the light source and filter set used.
3. Move the sample approx 1 μm for fluorescence image. The depth of focus of a 1.4 NA objective is quite small, so there can be a focus difference between the

reflectance image acquired at 450 nm and the fluorescence image acquired at 690 nm.
4. Choose the fluorescence filter set.
5. Acquire and save the fluorescence image with shuttered light. The light source needs to be shuttered to control the fluorescence exposure time and prevent photobleaching. Typical integration times are more than 1 s.

The components of one system capable of such measurements are as follows.

1. Zeiss Axiovert 100 microscope.
2. Sutter 175-W Xe lamp.
3. Photometrics CoolSnapHQ charge-coupled device camera.
4. Prior automated xyz stages.
5. Sutter automated filter wheels for excitation and collection. The excitation wheel must have a shutter.
6. Polytec PI PIFOC® piezoelectric drive for fine focus.
7. ×63 and ×100 oil immersion objectives (1.4 NA).
8. MetaMorph™ software from Universal Imaging for system control and image capture.
9. Glass-bottomed 96- or 384-well plates.
10. Reflectance excitation filter: 400- to 460-nm filter with 10- to 40-nm bandpass.
11. Fluorescence filter set.

As described, this system is capable of acquiring a pair of reflectance and fluorescence images in <5 s. If a ×63 objective is used, one can image 100 or more particles. A good sample of any assay point comes with averaging the fluorescence from 50 to 100 particles; thus, if multiple codes are used, multiple images are required. Image acquisition in assays requires the coordinated use of all the listed components. Software packages such as MetaMorph can automate the control of all items and perform automatic focusing.

Any fluorescent dye can be used with the particles; however, the differential reflectance of the metals means that the banding pattern will appear in the fluorescent image of any dye emitting below 600 nm. Cy5 is the best dye to use, because the absorption is well away from the reflectance imaging wavelengths and thus will not photobleach during focusing. The emission of Cy5 is also well above 600 nm and, therefore, the banding pattern will not appear. Fluorescein is the dye most likely to photobleach during focusing, because its absorption spectrum is close to or contains the reflectance image wavelengths. The dichroic filters for Cy3 and Cy5 will reflect and transmit enough light for the reflectance image and an automatic dichroic filter changer is not necessary.

Both reflectance and fluorescence images of particles may be acquired on a standard glass microscope slide and using an upright microscope.

High-magnification oil immersion objectives give the best NBC detection and fluorescent sensitivity; however, they require a PIFOC microscope objective positioner for automatic focusing. For imaging of NBCs, ×63 .8NA objectives can be used and will reduce the complexity and expense of the system. The reduced resolution means a smaller number of codes can be distinguished, but the required focus accuracy is reduced considerably. Standard stepper motor-driven objective-focusing systems can be used with this objective, and the focus offset between reflectance and fluorescence can be eliminated.

3.7. Image Processing

Analysis of the reflectance and fluorescence images is carried out with a proprietary software package developed in-house at Nanoplex Technologies—BSee™ software—that rapidly decodes the identity of the particles imaged and with an extremely high level of accuracy. The automated image-processing solution for NBC images must meet the following criteria: It must handle multiple images, both in the sense of analyzing and coupling the reflectance (RFL) and fluorescent images (FL), and in the sense of amassing statistics from many RFL/FL pairs. It must work over a large range of image conditions and accept some degree of blurring and other aberrations in the images. The software is designed to consider only isolated rods. Coincident rods and large clumps are rejected, because they can confound rod identification and increase the error in fluorescence quantitation.

Processing begins with the reflectance image. A high-pass filter is first applied to the reflectance image. This kernel has the effect of both enhancing the edges of the rods and separating those rods that are in close proximity. The image noise is assessed and combined with the median background level to determine the threshold intensity level that must be applied for proper segmentation—a method of isolating objects in a binary image. Applying a threshold cutoff to the image produces a binarized image whose nonzero pixels correspond to NBCs and whose zero pixels correspond to background.

The connected nonzero regions in the binarized image are designated as "blobs" or potential NBCs. Using principal component analysis (PCA) ***(10)***, the original gray-scale image pixels in each blob, corresponding to a single NBC, are used to determine the medial axis of the NBC. In PCA analysis, the first principal axis closely matches the best-fit line used to extract the intensity level along the length of the NBC.

The intensity profile is used to match the NBC to a library of striping profiles or "flavors." Two main scoring algorithms are used. The first is based on correlating the intensity profile to synthetic profiles in the library. The second method divides the intensity profile into a given number of segments and determines the metal choice for each segment. Once the flavor of an NBC has been identified, the fluorescent intensity of the NBC is assessed using the stored fluorescent

image. More specifically, those same pixel coordinates that are used to highlight the NBC image in the reflectance image are used with modification to quantify fluorescent intensity. The software generates mean and median background subtracted values for the fluorescence. Data are then accumulated over all NBCs of the same flavor. For instance, fluorescent values corresponding to all rods with the same striping pattern are averaged together. The data are output visually in spreadsheet form as well as to a file in a text format appropriate for Excel. More information about licensing the software can be obtained from Nanoplex Technologies.

4. Note

1. Always prepare the EDC/NHS solution fresh every day.

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