Chapter 2

Multiplexed Detection of Oligonucleotides with Biobarcoded Gold Nanoparticle Probes

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Abstract

Applications in a variety of fields rely on the high-throughput ultrasensitive and multiplexed detection of oligonucleotides. However, the conventional microarray-based techniques that employ fluorescent dyes are hampered by several limitations; they require target amplification, fluorophore labeling, and complicated instrumentation, while the fluorophore-labeled species themselves exhibit slow binding kinetics, photo-bleaching effects, and overlapping spectral profiles. Among the emerging nanomaterials that are being used to solve these problems, oligonucleotide–gold nanoparticle conjugates (Oligo-AuNPs) have recently been highlighted due to their unique chemical and physical properties. In this chapter, a detection scheme for oligonucleotides that utilize Oligo-AuNPs is evaluated with multiple oligonucleotide targets. This scheme takes advantage of the sharp melting transitions, intense optical properties, catalytic properties, enhanced binding properties, and the programmable assembly/disassembly of Oligo-AuNPs.

Key words: Oligonucleotide, Multiplexing, Gold nanoparticle, Detection, Sensing, Biomolecule, DNA, RNA

1. Introduction

The dramatic development of genomics and proteomics in the last several decades has attracted biologists to investigate how the two fields merge together at the molecular level. For example, the desire to understand how proteins are expressed and what genes cause the expression has strongly motivated researchers to investigate proteins or genes in vivo or in vitro under different experimental conditions. To accomplish this task, they have taken advantage of current molecular biology techniques such as mass spectrometry (1), tap-tags (2), ELISAs (3), microarrays (4), and blottings associated with gel electrophoresis. However, the need to develop alternative assays that are rapid, sensitive, and selective

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is growing significantly since the progress of the current research is hampered by the low-throughput, high cost, slowness, and, in certain cases, limited reproducibility of the conventional methods.

Emerging nanotechnologies are considered to be promising to solve these problems. Due to their unique and characteristic properties that have not been observed in macroscopic worlds previously and that excel the conventional materials in efficiency, nanomaterials have been intensively investigated and utilized in a variety of applications in diagnostics and therapeutics (5–7). Among the large number of nanomaterials that have been synthesized, oligonucleotide–gold nanoparticle conjugates (Oligo-AuNPs) have been used as probes in numerous detection schemes for biomolecule targets (8). The unique chemical and physical properties of Oligo-AuNPs that stem from their "inorganic nanoparticle core and densely packed oligonucleotide shell" structure offer high sensitivity and selectivity in such applications. To begin with, a brief introduction of oligonucleotides, AuNPs, and Oligo-AuNPs is given as follows.

1.1. Oligonucleotide

An oligonucleotide is a short nucleic acid, or polymer of nucleotides, with typically fewer than 100 bases. Although it is possible to cleave longer DNA or RNA strands to obtain them, oligonucleotides often refer to "synthetic" ones synthesized by polymerizing individual nucleotide precursors utilizing phosphoramidite chemistry. Because their chemical identity is the same as that of longer DNA or RNA except in terms of length, oligonucleotides have most of the same chemical and physical properties; they display a UV absorbance at 260 nm, form duplexes via base pairing, have binding interactions with proteins, and form monomolecular structures, such as G-quadruplexes. In addition, a variety of modified bases and nucleotide linkages (e.g., 2′-O-methylated RNA bases, phosphorothioate-modified phosphate backbones, and locked nucleic acids (LNA)) have been developed to control their properties, especially to enhance their stability.

One of the most important properties of oligonucleotides is reversible duplex formation. Based upon the pairing of the four bases (adenine (A)-thymine (T) and guanine (G)-cytosine (C)) via hydrogen bonds, an oligonucleotide sequence can reversibly bind or hybridize to the complementary sequence. During the hybridization process, its absorbance at 260 nm decreases due to hypochromism. When heated, however, the duplexed oligonucleotides dehybridize into single strands with an increase in absorbance at 260 nm. If this dehybridization, or "melting" process, is monitored as a function of temperature using UV–vis spectroscopy (at 260 nm), a broad curve (full width at half maximum (FWHM) ~10°C) is observed, whose midpoint is considered to be the melting temperature (T_m) of the duplex system. The melting temperature, which is the representative value of the stability of

the duplex pair, can be controlled by changing the salt concentration of the solution, the length of the sequence, or the number of mismatched bases.

Synthetic oligonucleotides, terminally modified with functional chemical moieties such as thiols, amines, carboxyls, fluorophores, quenchers, or biotin groups, are getting more and more widely utilized in nanotechnology applications, including microarrays (9), DNA origami (10), nanomaterials assembly (7, 8, 11), and so on. In this chapter, we cover how oligonucleotides that are conjugated with nanomaterials, specifically gold nanoparticles, play a significant role as a "smart" material based upon the aforementioned properties.

1.2. Gold Nanoparticles: Chemical and Physical Properties

Colloidal gold nanoparticles (AuNPs) have been investigated by many researchers because of their unusual properties that include relatively high stability, low toxicity, catalytic activity, surface plasmon resonance (SPR), enhanced Raman signal, and facile chemical tailorability. Also, these structures can support multiple functionalities on their surface. AuNPs with diameters between 2 and 250 nm can be prepared in aqueous media in relatively monodisperse forms using a variety of synthetic methods (12, 13). The unique and intense colors associated with these AuNPs are due to their SPR. This SPR is observed in the visible region of the spectrum and is due to the collective oscillations of the free electrons in the conduction band interacting with the electromagnetic field of the incoming light. In solution, monodisperse AuNPs (~15 nm in diameter) exhibit a red color indicative of a surface plasmon absorption centered at 520 nm. Since AuNPs, especially those stabilized with citrate, are charged particles, they are exceedingly sensitive to changes in solution dielectric. For example, the addition of sodium chloride (NaCl) shields the surface charge of these AuNPs and leads to a concomitant decrease in interparticle distance and eventual particle aggregation. A solution containing coalesced AuNPs appears purple or blue in color, corresponding to a characteristic dampening and red-shifting of the SPR of these particles, to around 600 nm. This distance-dependent optical property forms the basis of certain colorimetric detection schemes that employ nanoparticle–biomolecule conjugates as probes.

1.3. General Properties of Oligo-AuNPs

An oligonucleotide–gold nanoparticle conjugate (Oligo-AuNP) is a two-component system comprised of thiolated-oligonucleotide strands and their nanoparticle scaffold. The properties of these inorganic nanoparticle core/organic biomolecular ligand hybrids stem from not only each component of the hybrids but also synergistically from the combination of those two materials. The deep red color exhibited by Oligo-AuNPs in aqueous media is from the SPR of the gold nanoparticles, which shows a narrow absorption in the visible range around 520 nm. However, the

chemical recognition abilities of these structures are derived from the oligonucleotide shell. When two complementary Oligo-AuNPs are combined, they form three-dimensional networks through DNA duplex interconnects and an effective shift of the SPR (50 nm or more) takes place with a concomitant red-to-purple color change (14). Due to the reversible hybridization properties of oligonucleotides, however, the assembled Oligo-AuNPs disassemble at an elevated temperature (or reduced salt concentration) returning back to red. Importantly, melting analyses of the hybridized particle aggregates show sharper melting transitions (FWHM ~2°C) than free DNA duplexes (15). The sharpness of this melting transition is explained by a cooperative mechanism that originates from the presence of multiple DNA interconnects between the Oligo-AuNPs and the melting cascade that results as counter ions are rapidly released from the aggregate (15, 16). Further, Oligo-AuNPs exhibit particle size-dependent melting properties and enhanced binding properties, which are the representative synergetic results of cooperative interactions between the AuNP and surrounding oligonucleotides (17, 18).

In addition, Oligo-AuNPs are chemically, physically, and biologically stable, and nontoxic. While the inertness of AuNPs is well known, the biological in vivo or in vitro stability of Oligo-AuNPs had remained a question until recently. Unlike free oligonucleotide strands, those on AuNPs are resistant to enzymatic degradation by DNase (19). This high biological stability is due to the densely packed oligonucleotides on the AuNP surface, which cause steric inhibition of the enzymatic cleavage reaction. This increased resistance to nuclease degradation plays a significant role in the therapeutic applications of these structures.

1.4. Biobarcoded Oligonucleotide–Gold Nanoparticle Conjugate Probes

Biobarcode assays are characterized by their ultrahigh sensitivity and multiplexing capability for the detection of oligonucleotides or proteins. In case of oligonucleotide targets, Oligo-AuNP probes are hybridized to oligonucleotide-functionalized magnetic microparticle (MMP) probes using the target sequence as a linker; these complexes are then separated magnetically for subsequent release of the oligonucleotides from the Oligo-AuNP probes (see Fig. 1.). These released oligonucleotides or "biobarcodes" are quantitatively analyzed by the scanometric assay. The high sensitivity of the biobarcode assays is believed to originate from four reasons: (1) Because the biobarcode assay can be considered as a pseudo-homogeneous assay, the binding equilibrium of the target and the particle probes can be increased by increasing the probe concentration. (2) The dense loading of oligonucleotides (biobarcodes) on the AuNPs was found to increase the equilibrium binding constant of the Oligo-AuNP up to two orders of magnitude higher than that of unmodified free DNA (18). (3) Due to the dense loading of oligonucleotides (biobarcodes) on AuNPs, one binding event of the target sequence to the MMPs

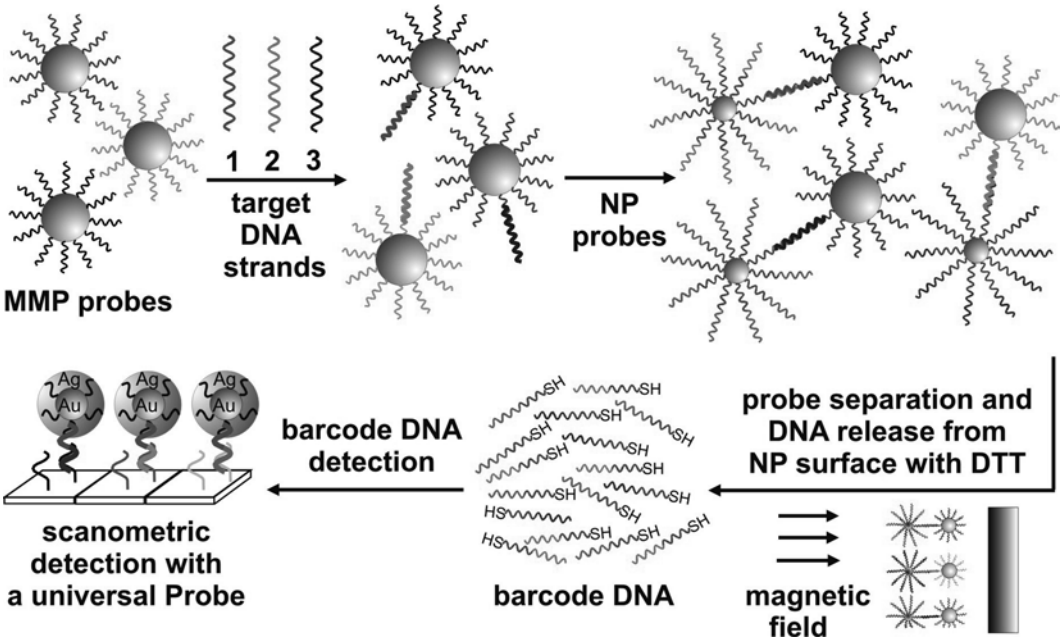

Fig. 1. Multiplexed biobarcode assay for oligonucleotide target detection. Reproduced from ref. 23 with permission from John Wiley & Sons, Inc.

and the AuNPs generates hundreds of biobarcode strands, leading to literally "hundreds of times" the target amplification. (4) Finally, these biobarcodes, as surrogate targets, are further amplified by silver enhancement during the scanometric assay. Except the second reason (2), biobarcode assays for protein targets also have the same reasons for the ultrahigh sensitivity, where antibodies on the particle probes play a role in binding the targets.

This chapter will discuss the general protocol of the biobarcode assay for oligonucleotide targets in a multiplexed form. In brief, it covers the synthesis of gold nanoparticles and their conjugates with oligonucleotides, the preparation of MMP probes, the main biobarcode assay procedure, and final scanometric assay. As mentioned above, the fundamental principles of the biobarcode assays for the detection of protein targets is very similar to what is described in this chapter for the oligonucleotide targets, except for the probe preparation that includes the conjugation of the particles with antibodies.

2. Materials

2.1. Gold Nanoparticle Synthesis

1. Gold (III) chloride trihydrate.
2. Ultrapure water (>18.2 MΩ cm).

3. Trisodium citrate.
4. Magnetic stirrer.
5. Stir bar (egg-shaped).
6. Round bottom flask.
7. Reflux condenser.
8. Heating mantle.
9. Temperature controller.
10. Concentrated hydrochloric acid (HCl, 37%).
11. Concentrated nitric acid (HNO, 70%).
12. Nylon filter (pore diameter = 0.45 μm).

2.2. Oligo-AuNP Conjugate Synthesis

1. Thiol-oligonucleotide of a given sequence (HPLC-purified) (see Note 1).
2. NAP-5 Sephadex column.
3. Dithiothreitol (DTT).
4. UV–vis spectrometer.
5. Sodium dodecyl sulfate (SDS) solution: 1 wt%
6. Phosphate buffer: 100 mM sodium phosphate ($Na_2HPO_4 + NaH_2PO_4$), pH = 7.4.
7. Sodium chloride solution: 2.0 M NaCl.

2.3. MMP Preparation

1. Amino-functionalized MMPs (2.8 μm in diameter; Invitrogen, Carlsbad, CA).
2. Succinimidyl-4-(*p*-maleimidophenyl) butyrate (SMPB).
3. Coupling buffer: 0.2 M NaCl, 100 mM sodium phosphate, pH = 7.0.
4. Passivation buffer: 0.15 M NaCl, 150 mM sodium phosphate, pH = 8.0.
5. Separation magnets (sample volume ≥1 mL).
6. Sulfosuccinimidyl acetate (Sulfo-NHS-acetate).
7. Ammonium sulfate ($(NH_4)_2SO_4$).
8. Oligonucleotides (3′-thiol MMP-sequences).
9. Anhydrous dimethyl sulfoxide (DMSO).

2.4. Multiplexed Biobarcode Assay for DNA Targets

1. AuNPs (30 nm in diameter, Ted Pella, Inc., Redding, CA).
2. Assay buffer: 0.2 M NaCl, 0.1% Tween 20, and 10 mM sodium phosphate, pH = 7.2.
3. Separation magnets (sample volume ≥1 mL).
4. Scanometric buffer: 0.5 M NaCl, 0.01% Tween 20, and 10 mM sodium phosphate, pH = 7.2.

5. Verigene Reader System (Nanosphere, Northbrook, IL).
6. A 96-well-plate magnet.
7. Oligonucleotides (target sequences, 5′-thiol AuNP-sequences).

2.5. Scanometric Detection of DNA Targets

1. *N*-Hydroxysuccinimide (NHS) ester-activated Codelink slides (SurModics Inc., Eden Prairie, MN).
2. Printing buffer: 150 mM sodium phosphate, 0.01% SDS, pH = 8.5.
3. Capture oligonucleotide (amine-modified) and target oligonucleotide (unmodified).
4. Passivating solution: 0.2% SDS.
5. Spin dryer.
6. Two-well manual hybridization chambers (Nanosphere, Northbrook, IL).
7. Temperature-controllable incubator.
8. Scanometric buffer: 0.5 M NaCl, 0.01% Tween 20, 10 mM sodium phosphate, pH = 7.2.
9. Verigene Reader System (Nanosphere, Northbrook, IL).
10. Silver enhancing solutions (Nanosphere, Northbrook, IL).
11. Formamide.
12. Microarrayer.
13. GenePix Pro 6 software (Molecular Devices).

3. Methods

3.1. Gold Nanoparticle Synthesis

1. Clean all glassware with aqua regia (3:1 HCl:HNO_3, see Note 2). Rinse the glassware with 18.2 MΩ cm ultrapure water and dry in an oven prior to use.
2. Bring an aqueous solution of $HAuCl_4$ (1 mM, 500 mL) to reflux while stirring.
3. Rapidly add 50 mL 38.8 mM trisodium citrate solution to the refluxing solution.
4. Observe that the solution quickly changes color from pale yellow to deep red.
5. After waiting 15 min, allow the mixture to cool to room temperature and subsequently filter it through a 0.45 μm nylon filter.
6. Characterize the colloid using UV–vis spectroscopy (see Note 3).

3.2. Oligo-AuNP Conjugate Synthesis

1. Dissolve the lyophilized thiol-modified oligonucleotides (~40 nmol) in 0.1 M dithiothreitol (DTT) solution in phosphate buffer (0.17 M sodium phosphate, 100 μL) for 30 min (see Note 4).
2. Purify the deprotected oligonucleotides from excess DTT using a NAP-5 column. Apply less than 300 μL of the oligonucleotide–DTT mixture to a NAP-5 column, and elute about 1 mL, collecting about 4–5 drops at a time in separate microtubes.
3. Measure the amount of DNA from each aliquot by UV–vis spectroscopy and add it to AuNP solution (see Note 5).
4. Bring the solution to 0.3 M NaCl, 10 mM sodium phosphate, and 0.01% SDS, pH = 7.4 (see Note 6).
5. Typically, the system is allowed to equilibrate at least for a couple of hours.
6. Remove the excess DNA by repeated centrifugation (16,000 × *g*, 25 min for 13 nm AuNPs; 12,000 × *g*, 10 min for 30 nm AuNPs) and wash with a buffer solution (the concentration of NaCl that you will use in the subsequent assay, 10 mM phosphate, 0.005% Tween 20, pH = 7.4) (see Note 7).
7. Store the Oligo-AuNP probes at 4°C prior to use (see Note 8).

3.3. MMP Probe Preparation

1. Wash the MMPs (30 mg/mL, 1 mL) twice with anhydrous dimethyl sulfoxide (DMSO, 1 mL).
2. Prepare a solution of SMPB (50 mg) in DMSO (15 μL) prior to the reaction (see Note 9).
3. Add the SMPB/DMSO solution to the magnetic beads.
4. Allow the reaction between the primary amino group of MMPs and the *N*-hydroxysuccinimide (NHS) ester of SMPB to proceed for 4 h with gentle shaking at room temperature.
5. Separate the beads magnetically and wash them three times with DMSO (10 μL) and two times with coupling buffer (10 μL).
6. Reduce the disulfide bonds in all 3′-thiol MMP-sequences using DTT prior to mixing with the SMPB-activated MMPs (see Note 10).
7. Prepare solutions (5 μM) of each of the freshly cleaved oligonucleotides in coupling buffer.
8. Add 300 μL of each oligonucleotide to each of the washed SMPB-activated magnetic beads.
9. Allow the reaction between the maleimide group and the thiol-oligonucleotide to proceed at 20°C for 1 h under constant vortex.

10. Place the oligo-functionalized MMPs on a magnet. Remove the supernatant, and wash the beads twice with coupling buffer and then twice with passivation buffer.
11. Use the supernatant to determine the coupling efficiency. Measure the absorbance at 260 nm and compare it with that before the oligonucleotide-functionalization of MMPs (see Note 11).
12. Passivate the surface of the MMP probes by adding a freshly prepared solution of sulfo-NHS-acetate (100 mg) in passivation buffer (40 μL) (see Note 12). Allow the passivation process to proceed for 30 min at room temperature with mild shaking.
13. Wash the MMP probes twice with passivation buffer, twice with assay buffer, and store them at 4°C in assay buffer at a concentration of 30 mg/mL.

3.4. Multiplexed Biobarcode Assay for DNA Targets

1. Prepare Oligo-AuNPs by conjugating oligonucleotides with the 30 nm-AuNPs (see Note 13) and finally redisperse them in assay buffer ([Oligo-AuNP] ~ 5 nM).
2. Prepare MMP multiplexing solution by combining all four MMP probes in assay buffer (final total MMP probe concentration is 1.56 mg/mL).
3. Begin the assay by mixing assay buffer (150 μL), MMP multiplexing solution (40 μL), and the appropriately mixed target solution (10 μL).
4. Heat the mixture at 45°C for 30 min and 25°C for 3 h under constant vortex to allow hybridization between the MMP probes and the target oligonucleotides (see Note 14).
5. Plate the reaction tube on a 96-well-plate magnet and wash the MMP–target complexes twice with assay buffer (200 μL).
6. Prepare Oligo-AuNP probe solution composed of all four NP probes (NP multiplexing solution) by diluting equal volumes of each NP probe (5 nM) in assay buffer containing formamide (15%) to a final total NP concentration of 200 pM (see Note 15).
7. Add the Oligo-AuNP multiplexing solution (50 μL) to the MMP–target complexes and allow hybridization to proceed at 25°C for 75 min under vortex.
8. Wash the mixture seven times with assay buffer (200 μL) using a 96-well-plate magnet to remove nonspecifically bound Oligo-AuNPs and any free barcode oligonucleotides.
9. Release the barcode oligonucleotides from the Oligo-AuNP probes by the addition of DTT (75 μL, 0.5 M) in scanometric buffer.

10. Collect the released barcode oligonucleotides from the mixture by removing MMPs using a magnet and analyze them quantitatively by the scanometric DNA detection method (see Note 16).

3.5. Scanometric Detection of DNA Targets

1. Dissolve amine-terminated capture DNA sequence (5′ H_2N-DNA sequence 3′) in printing buffer. The final concentration of the capture DNA sequence is 100 μM (see Note 17).
2. Print the DNA spots on a Codelink slide using a microarrayer. Try to keep the humidity in the arrayer as low as reasonably possible (see Note 18).
3. Hydrolyze the unreacted NHS groups of the slides or "passivate" the slides with passivating solution at 50°C for 20 min.
4. Spin-dry the slides.
5. Inject target oligonucleotides (20 μL) into hybridization chambers attached to a microarray slide prepared as above.
6. Hybridize the oligonucleotides to the capture strands on the slide in an incubator at 60°C for 15 min, at 37°C for 30 min, and at 25°C for 15 min with mild shaking (see Note 19).
7. Disassemble the chambers and rinse the slides copiously with scanometric buffer, spin them dry, and reassemble them with an unused hybridization chamber.
8. Introduce the Oligo-AuNP chip-probe solution (20 μL, [Oligo-AuNP] = 500 pM) into the hybridization chambers and allow the probes to hybridize at 37°C for 45 min (see Note 20).
9. Disassemble, wash three times using scanometric buffer, and spin-dry the hybridization chambers.
10. Perform the silver enhancement of the AuNPs by applying the silver enhancing solution (2 mL; Nanosphere, Northbrook, IL) on the dried chips for 3 min. Terminate the reaction by washing the slides with ultrapure water (18.2 MΩ cm) (see Note 21).
11. Spin-dry the slides and image them with the Verigene Reader System (Nanosphere, Northbrook, IL), which records the scattered light from the developed spots. Alternatively use a flatbed scanner to read out the scattering signal. Examples of the data obtained during readout are shown in Fig. 2.

4. Notes

1. Four exemplary sequences (Au1–Au4) for the AuNP probes used in biobarcode assays are given as an example.

   Au1: 5′ HS-TACGAGTTGAGAATC-TACCACATCATC CAT 3′

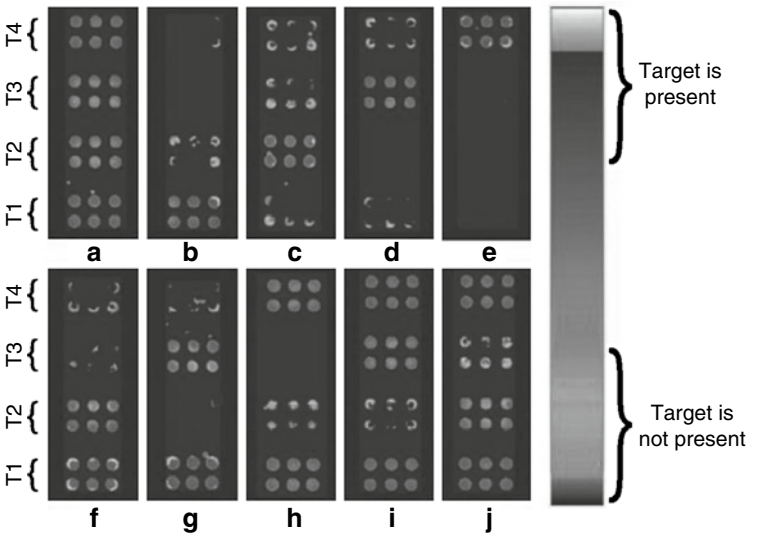

Fig. 2. Scanometric detection of the biobarcode-oligonucleotides released from the 30-nm Oligo-AuNP probes for ten different samples. (**a**) All targets are present; (**b**) T1; (**c**) T2; (**d**) T3; (**e**) T4; (**f**) T1 and T2; (**g**) T1 and T3; (**h**) T1 and T4; (**i**) T1, T3, and T4; and (**j**) T1, T2, and T4. The gray-scale images from the Verigene Reader System were converted into colored ones using GenePix Pro 6 software (Molecular Devices). The diameter of the spots on the chip is 200 μm. Reproduced from ref. 23 with permission from John Wiley & Sons, Inc.

Au2: 5′ HS-TACGAGTTGAGAATC-CTGATTACTATT GCA 3′
Au3: 5′ HS-TACGAGTTGAGAATC-TTGTTGATACTG TTC 3′
Au4: 5′ HS-TACGAGTTGAGAATC-TGCATCCAGGTC ATG 3′

2. Aqua regia is an extremely powerful oxidizing solution which is the only acidic solution that can be used to dissolve gold. Use extreme caution when working with aqua regia, because it generates harmful chlorine (Cl_2) and nitrogen oxide (NOx) gases and can cause severe tissue damage.
3. The characteristic SPR band of monodispersed particles is located around 520 nm. Particles with a more uniform size distribution have narrower absorption bands. Note that aggregated 13 nm gold nanoparticles, as discussed above, display a flattened and red-shifted absorption peak at approximately 600 nm.
4. Usually, the DNA sequences are 15- to 20-base pairs in length, complementary to a DNA target of interest, and modified with either 3′ or 5′ terminal sulfur-containing groups in the form of monothiols, cyclic disulfides (20), or trithiols (21). The terminal monothiol group of each oligonucleotide

is protected in the oxidized form (disulfide), which needs to be reduced by DTT to the monothiol form before reaction with gold nanoparticles.

5. The final oligonucleotide and AuNP concentrations are ~4 μM and ~6 nM, respectively.
6. The final concentrations of sodium phosphate (10 mM), sodium chloride (0.3 M), and SDS (0.01%) are achieved by adding the appropriate amounts of the concentrated solutions described in Subheading 2.2 to the oligonucleotide–gold nanoparticle mixture. The number of oligonucleotides per gold nanoparticle (15 nm in diameter) can be increased (~50–100 strands per particle) by increasing the NaCl concentration (from 0.1 to 1 M NaCl, respectively). Sodium ions screen the repulsions between the negatively charged oligonucleotide strands. The oligonucleotide loading also increases when the sequence has a polyethylene glycol (PEG) unit near the thiol group, or the mixture of oligonucleotides and gold nanoparticles is sonicated (22).
7. The removal of the excess DNA based upon centrifugation is repeated typically four times. More centrifugation steps can be performed when high purity is required, depending on the experimental purposes.
8. Oligo-AuNPs are stable and biochemically active for about 2 months when stored at 4°C.
9. Caution should be taken to prevent exposure of the solution to light.
10. The reduction of the disulfide bonds by DTT and the purification of the thiol-oligonucleotides are performed following the procedure described in Subheading 3.2. Four thiolated sequences (M1–M4) are given as an example; the sequence of these strands could be modified according to the experimental purposes [23]. The A_{10} portion near the thiol group is a spacer and does not participate in hybridization.

    M1: 5′ ATAACTGAAAGCCAA-A_{10}-SH 3′

    M2: 5′ TCTTCCGTTACAACT-A_{10}-SH 3′

    M3: 5′ TCCAACATTTACTCC-A_{10}-SH 3′

    M4: 5′ TTATTCCAAATATCTTCT-A_{10}-SH 3′
11. Typically, there are on average 3×10^5 oligonucleotides per MMP.
12. Sulfo-NHS acetate acylates primary amino groups in basic media.
13. The conjugation of the oligonucleotide with the AuNP probe is performed following Subheading 3.2, except that the final

NaCl concentration is 0.2 M. Four thiolated sequences (Au1–Au4) are given as an example; these sequences could be modified according to the experimental purposes (23). The "TACGAGTTGAGAATC" portion is common to all the sequences and is used for hybridization with the Oligo-AuNP chip-probes in the scanometric assay.

Au1: 5′ HS-TACGAGTTGAGAATC-TACCACATCATCCAT 3′

Au2: 5′ HS-TACGAGTTGAGAATC-CTGATTACTATTGCA 3′

Au3: 5′ HS-TACGAGTTGAGAATC-TTGTTGATACTGTTC 3′

Au4: 5′ HS-TACGAGTTGAGAATC-TGCATCCAGGTCATG 3′

14. Four unmodified target sequences (T1–T4) are given as an example; the sequence of these strands could be modified according to the experimental purposes. The initial reaction temperature (45°C) was determined to completely dehybridize the target sequences from the oligonucleotides on MMPs, leading to better duplex formation at 25°C in the next step.

    T1: 5′ TTGGCTTTCAGTTAT-ATGGATGATGTGGTA 3′

    T2: 5′ AGTTGTAACGGAAGA-TGCAATAGTAATCAG 3′

    T3: 5′ GGAGTAAATGTTGGA-GAACAGTATCAACAA 3′

    T4: 5′ AGAAGATATTTGGAATAA-CATGACCTGGAT GCA 3′

15. Formamide increases the stringency at which hybridization occurs since it competes with the complementary DNA strand for hydrogen bonding interactions. The addition of 15% formamide to the reaction mixture resulted in optimal selectivity of the system.

16. The scanometric detection of DNA is performed following the procedure in Subheading 3.5.

17. Only use HPLC-purified oligonucleotides. Amine contaminants can reduce coupling.

18. Codelink Slides must be stored desiccated, because the NHS leaving group on the Codelink slide also reacts with H_2O, resulting in a decrease in the coupling efficiency of the amine-modified oligonucleotides. Before and after each spotting run, always wash the pins 3–4 times to be sure that there is no salt build-up on them.

19. The incubation temperatures were controlled to completely dehybridize the biobarcode sequences from the capture sequences on the chip at 60°C and to enhance the hybridization by gradually decreasing the temperatures (37 → 25°C). This process is called "annealing."

20. The hybridization temperature is determined empirically not to dehybridize the probes at too high temperature, nor to hybridize the probes nonspecifically at too low temperature.
21. The staining time needs to be optimized depending on the number of hybridized AuNP probes. Longer staining time than enough saturates the signal intensity, while too short staining time cannot enhance the low signal intensity enough to be visualized.

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