Synthesis and Application of FRET Nanoparticles in the Profiling of a Protease

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Combinatorial libraries

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**Synthesis and Application of FRET
Nanoparticles in the Profiling of a
Protease**

Fluorescent silica nanoparticles incorporating unique ratios of energy transfer dyes are synthesized and applied as colloidal barcodes to encode a microsphere-bound combinatorial peptide library. The affinity of the West Nile virus protease is profiled using this library with cleavage of the peptide detected by flow cytometry. The cleaved peptide substrates are sorted and then identified through decoding by confocal microscopy combined with spectral unmixing (see image).

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Synthesis and Application of FRET Nanoparticles in the Profiling of a Protease**

Lionel Marcon, Corentin Spriet,* Timothy D. Meehan, Bronwyn J. Battersby, Gwendolyn A. Lawrie, Laurent Héliot, and Matt Trau

1 Disease biomarker identification, pathogen detection, drug
2 discovery, and biomolecular profiling require assays utilizing
3 large numbers of molecules.^[1–3] These assays typically involve
4 screening chemical libraries to identify target molecules such as
5 antigens, nucleotides, or peptides. Split-and-mix synthesis has
6 emerged as a powerful strategy for creating enormous
7 combinatorial libraries, in which multiple products are
8 synthesized simultaneously on solid support microspheres.^[4]
9 The conventional strategy for encoding split-and-mix libraries
10 involves covalent binding of chemical “identifier” tags (e.g.,
11 nucleic acids, secondary amines, fluorophenyl ethers)^[5] to the
12 microspheres in parallel with the compound synthesis. How-
13 ever, there are many advantages associated with the use of
14 optical encoding, rather than chemical encoding, with the major
15 advantage being the ability to use flow cytometry for rapid
16 biomolecular analysis.

17 Several attempts have been made to combine fluorescence
18 encoding methods with solid phase library synthesis.^[6–9] These
19 methods generally use “pre-encoded” support microspheres,
20 that is, the supports are encoded before the library synthesis
21 proceeds. However, we recently introduced a method for
22 optically encoding solid phase libraries in parallel with, rather
23 than prior to, combinatorial synthesis (i.e., colloidal barcod-
24 ing).^[10–14] This unique method involves encoding solid support

microspheres (10–20 μm) with smaller silica reporter particles
(0.1–0.8 μm) in order to tag each step during library synthesis.
The reporters contain a single dye, or combinations of dyes,
which impart unique spectral signatures that can be decoded by
fluorescence microscopy to identify the compounds synthe-
sized.^[10,11] Colloidal barcoding has been successfully utilized to
produce a library of 100 encoded tripeptides^[10] with the
possibility of encoding a library containing 64 million chemical
species by incorporating combinations of only seven dyes into
the reporters.

After conducting a biomolecular assay with the solid phase
library, the support microspheres that bear bioactive library
members are sorted rapidly via a flow cytometer. Conventional
fluorescence microscopy can be used to manually read the
colloidal barcode on each support microsphere and requires
differentiation of up to seven different dyes incorporated within
the reporters that form the colloidal barcode. This necessitates
the use of multiple filters to limit the overlap in the excitation
and emission spectra of the reporters. An alternative strategy is
described here and involves incorporating different combina-
tions of energy transfer fluorescent dyes within the silica
reporters. By varying the concentrations of dyes, the reporters
can exhibit multiple colors with tunable emission signatures by
fluorescence resonance energy transfer (FRET) under a single
wavelength excitation. FRET only occurs when the emission
and absorption spectra of the donor and acceptor overlap
and when the molecules are separated by a critical distance
(1–10 nm). This is achieved in the reporter particles due to the
covalent localization of the fluorophores within a silica matrix.
This strategy has been demonstrated using polystyrene micro-
spheres^[15] and silica particles^[16] in other studies, however, has
not before been applied to optical encoding in combinatorial
chemistry. The traditional filter-based decoding approach lacks
the resolution required to decode the complex spectral
signatures produced by FRET reporters,^[17] however confocal
microscopy can be used to quantify the contribution of different
dyes in each reporter.^[18]

In this paper, we present a novel colloidal barcoding method
utilizing nanoscale FRET reporters decoded by confocal laser
scanning microscopy combined with spectral unmixing. This
strategy was implemented to encode a combinatorial peptide
library that was directed against a model protease (Figure 1).
Fluorescent silica reporters were synthesized through a
modified Stöber procedure that has been described in
detail elsewhere.^[19] Briefly, succinimidyl ester dyes Atto-488
(λ_{ex} 501/ λ_{em} 523 nm), Atto-550 (λ_{ex} 554/ λ_{em} 576 nm), and

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Supporting Information is available on the WWW under <http://www.small-journal.com> or from the author.

Figure 1. Principle of the colloidal barcoding method. a) Optically barcoded combinatorial peptide libraries synthesized on support microspheres and terminated with a dye (violet) are incubated with a protease (yellow). For clarity, aa stands for amino acid. b) The fluorescence of the terminal dye is detected by flow cytometry. Microspheres exhibiting a loss of the terminal dye are sorted. c) The hit microspheres are then decoded by confocal microscopy to identify the sequence of the attached peptide.

Atto-590 (λ_{ex} 594/ λ_{em} 624 nm) (Atto-Tec, Siegen, Germany) were covalently linked to a silane coupling agent (3-aminopropyl)-trimethoxysilane (APS, Aldrich, USA) forming an APS-dye conjugate. These three dyes were selected to enable FRET using a single 488 nm excitation source resulting from the spectral overlap between the donor's emission and the acceptor's excitation (Figure 2a). Silica particle formation was initiated by reacting the APS-dye conjugate with tetraethoxysilane (TEOS, Aldrich) and ammonia (NH_4OH). The resulting reporters were coated with a polyelectrolyte bilayer composed of poly(diallyldimethylammonium chloride) and poly(acrylic acid) (Aldrich) to promote their adhesion to the support microspheres.^[10,11]

As a first step in the synthesis of the encoded library, it was necessary to tune the synthesis of the reporters to optimize the size and homogeneity of the particles. This was crucial to the success of the encoding strategy as the reporters must both i) permit the incorporation of a sufficient concentration of dyes to be distinguishable by confocal microscopy and ii) be

Figure 2. a) Fluorescence spectra (provided by the supplier) of the dyes used in this study. Dashed lines and continuous lines represent the excitation and emission spectra respectively. b) Spectrally unique emission signatures of reporters measured for 1× series.

sufficiently small to maximize the number of reporters that could be attached to the support microspheres. Previous studies, indicating that the particle size and dispersity were functions of the concentration of NH_4OH used in the synthesis, were used as a guide in this investigation.^[20,21] Concentrations from 0.8 to 6.3 M resulted in reporter sizes ranging from 50 ± 3 nm to 850 ± 15 nm, respectively (see Supporting Information). A secondary nucleation was observed at concentrations greater than 4.2 M that resulted in an undesirable polydisperse particle size distribution and the formation of aggregates. Considering the initial decoding observations by confocal microscopy (data not shown), it was determined that the 120 ± 5 nm reporters synthesized using 4.2 M NH_4OH represented the best combination of high fluorescence signal and narrow size distribution.

To obtain distinguishable emission signatures, the three dyes were incorporated into the reporters at five molar ratios (0, 1×, 2×, 3×, and 5×). For example, a reporter having 1× of Atto-488, 2× of Atto-550, and no Atto-590 dye (code 1/2/0) was distinguishable from a reporter having 1× of Atto-488, 2× of Atto-550, and 1× of Atto-590 dye (1/2/1). To illustrate FRET between the three dyes within the reporters and that the signatures were distinguishable, the fluorescence emission spectra of the ratio 1× reporter series are presented in

Figure 2b. It is apparent, by comparing the spectra of the double (1/1/0) and triple-dyed reporters (1/1/1) with those of the single-dyed reporters (1/0/0, 0/1/0, and 0/0/1), that the emission of the donor Atto-488 dramatically decreased when associated with Atto-550 and/or Atto-590. Inversely, the emission of the acceptor moieties was shown to increase. A red-shift in emission, as noted in a previous study, was also observed here when the dyes were combined.^[15]

To evaluate the utility of the reporters to form a colloidal barcode, the reporters were loaded on 10 μm amine functionalized polystyrene microspheres (Tentagel, Rapp Polymere, Tübingen, Germany) in eleven different combinations of between three and seven codes (see composition in Supporting Information). Reference spectra for the three dyes alone were acquired from the single-dyed reporters (1/0/0, 0/1/0, and 0/0/1) using a Leica SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany). Utilizing these reference spectra as a calibration, the contributions of each dye to the composite fluorescence signature of multiple-dyed reporters were quantified using the spectral unmixing algorithm supplied with the acquisition LCS software.

The adhesion of the reporter particles to a microsphere is shown in the scanning electron microscopy (SEM) image (JEOL JSM 6400F, Tokyo, Japan) in Figure 3a and an example of the decoding of a microsphere bearing a combination of 4 reporters (5/1/1, 1/5/0, 1/1/5, and 5/1/0) is presented in Figure 3b–d. Analysis of the spectra of the four reporters presented in Figure 3d demonstrates that they are readily distinguishable. The spectral reproducibility of reporters attached to microspheres was assessed and found to have an average coefficient of variation equal to 5%. Decoding data (image and fluorescence spectra) of the most significant

combinations are given in the Supporting Information along with the experimental set-up.

Following characterization, the reporters were used to encode a combinatorial peptide library that was applied to profiling the affinity of the West Nile flavivirus protease (WNV-pro). WNV is a potentially lethal human pathogen found throughout the tropical world. The protease produced by the virus is required to cleave the precursor viral polyprotein into the individual functional proteins that are essential for viral replication.^[22] Previous studies by Chappell et al. and Shiryayev et al. defined the optimal WNV-pro substrates as Leu-Gly-X-X↓Gly-Gly-Ala-Ala where X is either Arg or Lys and the cleavage site is represented by ↓.^[23,24] These researchers demonstrated that the proteolysis was most profoundly affected by an alteration of the X-X dibasic motif.

On this basis, a library composed of 144 octapeptides was constructed via a split-and-mix method using natural amino acids by standard Fmoc chemistry in which the X-X position was encoded. The amino acids in the X-X positions were randomized from a pool of twelve amino acids to incorporate a representative of each significant category (acidic, basic, hydrophilic, and hydrophobic). The library and its encoding are described in the Supporting Information. Following the peptide synthesis, Atto-350 (λ_{ex} 350/ λ_{em} 423 nm), a dye excited in the UV range and with an emission spectrum different from those of the reporters, was covalently linked to the terminal amino acid of each peptide (Figure 4a).

The library was incubated with the WNV-pro for 1 h at 37 °C. Proteolysis was detected via flow cytometry (BD LSR II, Becton Dickinson, North Ryde, Australia) through the reduction of on-microsphere fluorescence of Atto-350

Figure 3. a) SEM image of a 10 μm microsphere with 120 nm silica reporters. b₁–₃) Contribution of each dye to the total fluorescence of the reporters after spectral unmixing. c₁, c₂) Composite image merging the three contributions and d) identification of each reporter's specific fluorescence code. These confocal images are represented using false colors for visual contrast.

Figure 4. a) Microsphere-bound peptide library produced by split-and-mix synthesis. Sequences in red lettering represent the substrates that were identified after decoding. b) Flow cytometry analysis after incubation of the peptide library with the WNV-pro. The population exhibiting a fluorescence decrease (shown in red) was collected for decoding. c) Corresponding barcodes.

(Figure 4b). A population exhibiting a fluorescence decrease of 80%, indicating proteolytic cleavage, was sorted and collected for confocal decoding. Four sequences, containing Arg and Lys in the two randomized positions, were identified in this population (indicated by red lettering in Figure 4a), in agreement with those substrates identified in the solution-based studies previously discussed.^[23,24] Representative composite spectra of the reporters encoding these randomized positions of the four sequences are presented in Figure 4c.

By utilizing d number of dyes at r number of ratios, ($r^d - 1$) unique optical codes can be generated.^[25] The use here of three dyes ($d = 3$) incorporated at five ratios ($r = 5$) therefore has the potential to generate a total of 124 unique codes according to ($5^3 - 1$). If one considers the creation of an encoded hexapeptide library in which every amino acid was randomized from a pool of all 20 natural amino acids, it can be calculated that only 120 codes would be required to distinguish all (20^6) 64 million unique peptides. Protease screening routinely utilizes peptides of no more than six amino acids in length.^[26] Therefore, our methodology can be directly applied to the profiling of newly discovered proteases with unknown affinities. However, encoding other chemical libraries (nucleotides, carbohydrates, etc.) might necessitate a greater number of FRET reporters. This could be accomplished by correlating emission spectra acquisitions with dye lifetimes. The direct determination of fluorescence lifetime is indeed one of the most direct measures of FRET.^[27] In addition, the use of multiple excitations would

extend the useful spectral range of the reporters enabling the creation of larger encoded libraries.

In summary, we reported here for the first time the synthesis of a combinatorial peptide library encoded using FRET reporter nanoparticles. Varying the ratios of energy transfer dyes served as an innovative route to produce unique spectral signatures. In addition, the use of confocal microscopy combined with spectral unmixing represents a major improvement in the interrogation of optically barcoded libraries by enabling the recognition of complex dye combinations. Our profiling of the West Nile virus protease was consistent with previous studies done with standard methods and represents an important step toward the practical implementation of colloidal barcoding. Such supports should find broad applications in gene expression studies, high-throughput screening, and medical diagnostics.

Keywords:

barcoding · confocal microscopy · fluorescence · nanoparticles · protease

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