Published in final edited form as:

Integr Biol (Camb). 2009 April ; 1(4): 311–317. doi:10.1039/b821775k.

Unbiased discovery of *in vivo* imaging probes through *in vitro* profiling of nanoparticle libraries

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Summary

In vivo imaging reveals how proteins and cells function as part of complex regulatory networks in intact organisms, and thereby contributes to a systems-level understanding of biological processes. However, the development of novel *in vivo* imaging probes remains challenging. Most probes are directed against a limited number of pre-specified protein targets; cell-based screens for imaging probes have shown promise, but raise concerns over whether *in vitro* surrogate cell models recapitulate *in vivo* phenotypes. Here, we rapidly profile the *in vitro* binding of nanoparticle imaging probes in multiple samples of defined target vs. background cell types, using primary cell isolates. This approach selects for nanoparticles that show desired targeting effects across all tested members of a class of cells, and decreases the likelihood that an idiosyncratic cell line will unduly skew screening results. To adjust for multiple hypothesis testing, we use permutation methods to identify nanoparticles that best differentiate between the target and background cell classes. (This approach is conceptually analogous to one used for high-dimensionality datasets of genome-wide gene expression, *e.g.* to identify gene expression signatures that discriminate subclasses of cancer.) We apply this approach to the identification of nanoparticle imaging probes that bind endothelial cells, and validate our *in vitro* findings in human arterial samples, and by *in vivo* intravital microscopy in mice. Overall, this work presents a generalizable approach to the unbiased discovery of *in vivo* imaging probes, and may guide the further development of novel endothelial imaging probes.

Introduction

In vivo imaging is emerging as a powerful tool to monitor biological processes in living organisms¹. *In vivo* probes can reveal cellular behavior and protein activity in their native context, amidst intact physiologic regulation at the level of interacting molecules, cells and organs. The discovery of novel *in vivo* probes, however, can be challenging. Typically, a pre-specified target protein (*e.g.*, one that is differentially expressed across cell types) is screened against a library of ligands (antibody, peptide or small molecule). In subsequent steps, ligands that emerge from a screen are often conjugated to a nanoparticle, such as a quantum dot or magneto-fluorescent nanoparticle, to confer imaging capabilities and favorable

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pharmacokinetics.² While this target-based approach has generated many *in vivo* probes, it can only be applied when there is *a priori* knowledge of a suitable candidate targeting ligand.

An alternative approach to discovering new imaging probes is systematically screening a library of imaging probes for useful phenotypes in cultured cells *in vitro*^{3–5} (i.e., a phenotype-based, as opposed to a target-based approach). This approach does not rely upon pre-specified imaging targets, and by analogy to forward genetic screens, can lead to the discovery of imaging probes that act through novel mechanisms and reveal new biology. However, this approach presumes that *in vitro* cell lines (typically single samples) can serve as faithful surrogate models for complex *in vivo* processes; in light of data suggesting that *in vitro* phenotypes can change rapidly upon cell culture⁶, the idiosyncrasies of a particular cultured cell line may limit the ultimate *in vivo* applicability of probes arising from these screens.

We sought to develop a more generalizable, robust approach to the discovery of *in vivo* imaging probes in order to enable integrative studies of biology in intact organisms. We frame the problem of probe discovery as an exercise in class distinction: that is, a promising imaging probe is one that maximizes signal in a desired class of cells (target cells), while minimizing signal in an alternative class of cells (background cells). We screen a library of imaging probes for their phenotypes in multiple distinct samples of each class of cells (target *vs.* background), and utilize statistical metrics to select those probes that best discriminate between target *vs.* background cells. The rationale for profiling imaging probes across multiple isolates of target and background cells is twofold. First, we reason that individual samples in a class would differ broadly in many phenotypes, but would still share phenotypes common to their identity as either target or background cells. Second, we hypothesize that comparing imaging probe phenotypes across all members of the target class *vs.* across all members of the background class would identify probes that most robustly distinguish between these classes without being overly dependent on any single isolate. We also utilize low-passage, primary isolates of human cells (as opposed to immortalized cell lines), to attempt to minimize (but not eliminate) altered cellular phenotypes caused by cell culture.

Here, we illustrate this approach by screening for imaging probes with enhanced binding to vascular endothelial cells. Vascular endothelial cells line the luminal surface of blood vessels, and are critical mediators of vascular homeostasis, inflammation, and repair. Despite their importance in health and disease, there are currently limited options for imaging the endothelium^{3, 7}. We screened a library of small-molecule-modified magneto-fluorescent nanoparticles against a large number of primary cell isolates belonging to two general classes: endothelial cells (target class) *vs.* macrophages (background class; the unmodified nanoparticle localizes almost exclusively to macrophages⁵). We then calculated a metric that reflects the extent to which nanoparticles bind differentially to endothelial cells *vs.* macrophages, across all cell lines tested; permutation testing assigns statistical significance to these differences. We demonstrate that this approach yields small-molecule-modified nanoparticles with significantly enhanced binding to endothelial cells *in vitro* (while keeping macrophage uptake constant). Furthermore, we validate the *in vitro* findings using *ex vivo* incubation with human carotid artery specimens, and *in vivo* intravital microscopy in mice. These results may speed the development of a novel imaging probes for endothelial cells *in vivo*, and suggest that profiling libraries of imaging probes across multiple cell lines can be a generalizable method to discover new imaging probes.

Results

Synthesis of a focused library of small-molecule-functionalized nanoparticles

We began with a widely used imaging platform consisting of a superparamagnetic iron oxide core that is rendered biocompatible by a coating of cross-linked 10-kD dextran (cross-linked

iron oxide, or CLIO 8); the dextran was cross-linked by epichlorhydrin and aminated to form CLIO-NH₂. CLIO-NH₂ was conjugated with fluorescein isothiocyanate (FITC) to yield CLIO-NH₂-FITC nanoparticles, in the presence of citrate pH 8.0 and *N,N*-diisopropylethylamine as catalyst⁵ (Scheme 1); CLIO-NH₂-FITC can be imaged by magnetic resonance or fluorescence, and the FITC moiety also enables detection or localization of the nanoparticle by antibody-mediated analytical approaches. Unreacted amine groups on CLIO-NH₂-FITC can then be conjugated to small molecules, and the resulting conjugates screened for their patterns of cellular binding.

Using this approach, a 147-member nanoparticle library was previously synthesized and screened for binding of individual library members to various cell types *in vitro*⁵. Based on these initial data, we synthesized a sub-library of selected small molecule-modified nanoparticles in which 38 commercially available anhydrides (Supplementary Fig. S1) were conjugated to CLIO-NH₂-FITC. In brief, anhydrides were conjugated to CLIO-NH₂-FITC in the presence of bicarbonate at room temperature, followed by sodium hydroxide, and small molecule-nanoparticle conjugates were purified by size exclusion chromatography⁵ (Scheme 1). The number of conjugated anhydrides per nanoparticle was determined by reacting anhydride-conjugated nanoparticles with SPDP⁹. Upon treatment with the reducing agent TCEP, the release of pyridine-2-thione (P2T) can be quantitated by absorbance, and reflects the number of unconjugated amines remaining after anhydride coupling; each nanoparticle bears approximately 2 FITC groups and 24 anhydride derivatives.

***In vitro* profiling and data analysis**

Because the starting CLIO-NH₂-FITC nanoparticle is exclusively taken up by macrophages⁵, we sought to alter its natural targeting by modifying its surface with conjugated small molecules. We screened a library of small molecule-modified nanoparticles for increased endothelial cell binding, with little or no increase in macrophage binding (relative to CLIO-NH₂-FITC). To attempt to more broadly sample endothelial and macrophage states, we profiled the binding activity of the nanoparticle library against multiple distinct samples of vascular endothelial cells or macrophages *in vitro*. We selected primary human endothelial cell isolates derived from a range of vascular beds: (a) large caliber arteries, such as the aorta and iliac artery; (b) the pulmonary artery, which experiences lower blood pressures; (c) veins, such as saphenous and umbilical vein; (d) small terminal microvessels from skin biopsies. We also screened nanoparticles against primary human macrophages, in both resting and activated states. Using an immunoassay for the FITC moiety on each nanoparticle, we calculated the apparent concentration of library nanoparticles that bound to each cell type (in pM)¹⁰

Of the 38 conjugated nanoparticles screened, several appear to selectively increase endothelial, but not macrophage binding, compared to the starting CLIO-NH₂-FITC nanoparticle (Fig. 1; complete binding data are in Supplementary Table). To evaluate which nanoparticles show the best discrimination between endothelial and macrophage cell binding, a t-statistic was calculated for each nanoparticle that reflects the difference in nanoparticle binding across all endothelial samples *vs.* across all macrophage samples; the statistical significance of this t-statistic was evaluated by calculating permutation-based p-values¹¹. For instance, nanoparticles 16-6 and 16-3 exhibit increased binding to endothelial cells *vs.* macrophages ($p = 3 \times 10^{-3}$ and 2×10^{-4} , respectively) (Fig. 1). Figure 2 plots the binding of these nanoparticles to the individual endothelial and macrophage cell types, and illustrates the prominent increase in endothelial binding ($p = 0.0001$ by ANOVA) with no change in macrophage binding ($p = 0.4$ by ANOVA). Both 16-6 and 16-3 show increased binding across almost all of the diverse endothelial isolates tested (Fig. 2). Note that because endothelial cells and macrophages were screened independently and under different assay conditions, direct comparisons of the extent of binding to endothelial cells *vs.* macrophages for individual nanoparticles are not

straightforward to interpret based on screening data alone. Figure 2 depicts the concentration of 16–3 and 16–6 bound to each cell type normalized to the concentration of unmodified CLIO-NH₂-FITC bound to the identical cell type.

Probe validation in carotid artery samples and in vivo mouse vessels

An intravenously administered *in vivo* imaging probe will encounter many other cell types in addition to the endothelial and macrophage classes that were defined in our profiling experiments. To better assess how our small-molecule-modified nanoparticles partition amongst endothelial cells, macrophages and other cells in a more physiologic context, and to validate our *in vitro* findings, we next evaluated the localization of conjugates 16–6 and 16–3 in human carotid endarterectomy specimens. These specimens are obtained (under approval from an Institutional Review Board for human studies) from patients undergoing surgery to excise atherosclerotic plaque from their carotid arteries, and are anatomically intact samples of the innermost portion of the vessel wall (*i.e.*, immediately adjacent to the vessel lumen); they consist of endothelial cells, infiltrating macrophages, smooth muscle cells, and extracellular matrix proteins including collagen and elastin. Nanoparticle conjugates 16–6, 16–3, and the starting material CLIO-NH₂-FITC were incubated with human carotid endarterectomy samples *ex vivo* for 24 hours. Samples were then processed for immunofluorescence, and nanoparticles were detected using antibodies against the FITC moiety conjugated to their surface. We tested whether nanoparticles co-localized with endothelial cells (identified by antibodies against the endothelial antigen CD31) or macrophages (identified by antibodies against the macrophage marker CD68). Both 16–6 and 16–3 show marked immunofluorescence that co-localizes with CD31 expression, consistent with endothelial uptake; the starting material CLIO-NH₂-FITC shows only weak endothelial localization (Fig. 3A). All three nanoparticles show comparable macrophage uptake, as evidenced by CD68 co-localization (Fig. 3B). Significantly, we do not observe widespread uptake of 16–6 or 16–3 throughout the cellular components of any specimens, arguing that the increase in endothelial localization is not associated with non-specific uptake of nanoparticles into multiple cell types.

We next tested the localization of probe 16–6, which showed the best *in vitro* binding profile across all of our tested endothelial cells (Fig. 2), following intravenous injection in living mice. Six hours following intravenous injection, intravital microscopy of ear vessels was performed using the FITC channel to detect the FITC moiety on the surface of the injected nanoparticles. As a reference for the intravascular space, Angiosense-IVM 750 (a 250 kilo-dalton macromolecule that freely circulates while staying confined to the vessel lumen; Visen Medical) was injected together with nanoparticles, and imaged using the near-infrared channel. As expected, 16–6 localizes to groups of cells immediately adjacent to the vessel lumen consistent with endothelial cells; a smaller portion of the 16–6 signal appears unassociated with the vessels, consistent with residual macrophage uptake (Fig. 4A). In contrast, unmodified CLIO-NH₂-FITC shows a very different distribution; the bulk of CLIO-NH₂-FITC signal is found unassociated with vessels, and the regions closest to vessels are not marked by accumulations of nanoparticles (Fig. 4B).

Discussion

By profiling a relatively modest number of small-modified nanoparticles, we have identified nanoparticles with enhanced endothelial binding (and relatively unchanged macrophage uptake). Significantly, these nanoparticles also show enhanced endothelial localization in human carotid endarterectomy samples, and upon *in vivo* intravital microscopy of mouse ear vessels, validating the findings of our *in vitro* profiling. An intriguing next step would be to test the probes described here in disease models in which endothelial changes have been

described, including atherosclerosis, cancer, and autoimmune diseases. More generally, these probes may provide clues for the further development of both pan-endothelial and subtype-specific imaging probes. The heterogeneity of vascular endothelium has long posed a particularly challenging target for *in vivo* imaging¹²; endothelial cell subtypes have been defined by the vascular bed of origin (e.g., different arterial, venous or microcapillary beds), developmental stage, and presence or absence of disease¹³. A collection of endothelial imaging probes would enable a host of applications, including *in vivo* studies of vascular development and repair, targeted drug delivery to specific vascular beds, and dissection of the role of endothelial alterations in disease. The profiling approach described here can easily be extended to identify subtype-specific probes, by altering the definitions of target *vs.* background cells (e.g., tumor-derived *vs.* control endothelial cells).

Small-molecule modification of magneto-fluorescent nanoparticles comprises a modular system that is well-suited to such screening approaches. The starting nanoparticle used here (CLIO) is well validated as an imaging and nanosensing agent (with favourable pharmacokinetic and toxicity profiles)^{8, 14}, and its aminated form can be readily conjugated to a wide variety of small molecule functional groups. Small molecule-targeting ligands can confer molecular specificity, and offer multiple potential advantages over peptide- or antibody-based targeting approaches (including smaller overall particle size, greater multivalency, decreased immunogenicity, greater ease of synthesis and stability, and decreased cost). This approach is likely to become even more powerful as diverse and complex small-molecule libraries are synthesized that not only possess novel biological properties, but also have chemical “handles” for subsequent conjugation steps built into the library design¹⁵.

Overall, the work described here combines the synthesis of libraries of small molecule-modified nanoparticles with phenotypic, cell-based profiling across target and background cell types. By identifying probes whose binding activity extends across an entire class of cell lines (thus sampling members of a cell class broadly, and decreasing the dependence on any individual cell line), and incorporating statistical methods that compensate for multiple hypothesis testing, the profiling approach described here may greatly facilitate the discovery of *in vivo* imaging probes, even in the absence of a pre-specified molecular target. (Indeed, ongoing experiments seek to clarify the protein targets that mediate the localization of small molecule-modified nanoparticles such as 16–6, as well as the distribution of these targets in endothelial cells and other cells accessible from the intravascular space.) The potential power of this approach is underscored by the observation that the profiling and analytic approaches used here are reminiscent of those applied successfully to genome-wide gene expression studies of cancer, including the identification of genes whose expression best distinguishes between cancer classes¹⁶. More generally, a screening approach in which nanoparticle binding is profiled across multiple cell isolates can be widely applied to the unbiased discovery of imaging probes that distinguish between different cell states, whether these different states are defined on the basis of genetic differences, histologic cell type, state of differentiation, or presence or absence of disease; such probes would be powerful enabling tools in wide-ranging *in vivo* studies of fundamental mechanisms and disease biology.

Experimental Section

Nanoparticle synthesis

The starting nanoparticle (CLIO) is a monocrystalline magnetic nanoparticle consisting of a 3 nm core of $(\text{Fe}_2\text{O}_3)_n(\text{Fe}_3\text{O}_4)_m$, covered by a layer of 10-kD dextran. The nanoparticle has an overall size (volume weighted) in aqueous solution of 38 nm, an R1 of 21 mM sec⁻¹, an R2 of 62 mM sec⁻¹ (37°C, 0.5T) and has an average of 62 primary amines available for conjugation. Cross-linking of dextran by epichlorohydrin, and amination by reaction with ammonia to yield CLIO-NH₂ was performed as described⁸. Fluorescein isothiocyanate (FITC) was dissolved in

DMSO and reacted with CLIO-NH₂ to yield an average of 2 FITC moieties per nanoparticle. The final reaction product was purified on Sephadex G-25 columns and used for small-molecule conjugation.

Conjugation of small molecules

To conjugate anhydrides to CLIO-NH₂-FITC, 100 μ L of the anhydride (at 50 mM in DMSO) was added to 1 mg CLIO-NH₂ (200 μ L at 5.0 mg/mL) in citrate solution. Reactions proceeded for 2–4 hours at room temperature to maximize conversion of all amines. Unreacted small molecules were removed using Sephadex G-25 columns eluted with PBS buffer, pH 7.4. Materials were characterized by size measurements, relaxometry, amine content, and mass spectrometry ⁵.

Chemicals

Dextran to synthesize the CLIO nanoparticles was purchased from GE-Healthcare (Uppsala, Sweden). All other chemicals were purchased from Sigma-Aldrich and used as received.

Cell screening

Endothelial cell lines, media, and media components were purchased from Clonetics/Lonza, and maintained as recommended by the manufacturer. Primary human macrophages were obtained as described ⁵. Briefly, buffy coats were subjected to density centrifugation, and mononuclear cells plated in primary human monocyte medium. Following 7 days of culture, adherent cells were considered resting macrophages. Cells were treated with GM-CSF for 7 days to yield GM-CSF activated macrophages.

For screening, all cells were plated in 96 well plates (10,000 cells/well for macrophages, and 4,000 cells/well for endothelial cells). Cells were incubated with 0.1 mg/mL Fe for each of the modified CLIO derivatives for 4 hours in a humidified 37°C incubator in the presence of 5% CO₂. Following incubation, wells were washed 3x with PBS/0.1% BSA/0.05% Tween-20 wash buffer and analyzed via a FITC immunoassay as described ¹⁰.

Computational Analysis

Raw immunoassay data were collected as absorbance values as described ¹⁰, and converted to concentration of nanoparticles retained in the well based on a standard curve. Results for each nanoparticle were scored as the median of either 3 (endothelial samples) or 4 (macrophage samples) technical replicates.

To evaluate which nanoparticles best discriminate between endothelial and macrophage cell binding, a t-statistic was calculated for each nanoparticle that reflects the difference in nanoparticle binding across all endothelial vs. all macrophage cell lines; the statistical significance of this t-statistic was evaluated by calculating permutation-based asymptotic p-values; calculations were performed using the Comparative Marker Extraction module of the GenePattern software suite ¹¹.

Human Carotid Specimens

Human carotid endarterectomy specimens were obtained under an established protocol approved by the Massachusetts General Hospital Institutional Review Board. Tissue specimens from patients undergoing surgery were collected, rinsed in HBSS prewarmed to 37°C, divided into 3 sections, then incubated with 10 mg/kg of 16–6, 16–3, or CLIO-NH₂-FITC for 24 hours in a humidified 37°C incubator in the presence of 5% CO₂. After incubation, the specimens were washed, embedded in OCT, and sectioned for immunofluorescence staining. Adjacent serial sections were blocked, incubated with anti-human CD31 antibody (Dako, Glostrup,

Denmark), anti-human CD68 antibody (Dako, Glostrup, Denmark), or anti-FITC antibody (Invitrogen, Carlsbad, CA) for 1 hour, washed, then developed by incubation with the appropriate secondary antibodies conjugated to FITC or Texas Red (BD Pharmingen, San Jose, CA). Finally, sections were mounted with ProLong anti-Fade reagent (Invitrogen, Carlsbad, CA) to preserve fluorescence and analyzed via microscopy (Nikon 80i Eclipse equipped with a 512 Photometrics Cascade CCD). Images were captured under uniform exposures.

Intravital microscopy

Intravital microscopy was performed on C57/BL6 mice using an Olympus IV100 intravital laser scanning microscope (Olympus Corporation, Tokyo, Japan).¹⁷ In brief, mouse ears were treated with a local injection of TNF α (5 ng in 50 μ l) 24 hours prior to nanoparticle injection. Nanoparticles were injected at a concentration of 15 mg Fe/kg *via* the retroorbital intravenous route, together with 50 μ l of Angiosense-IVM 750 (to visualize the intravascular space). Mouse ears were imaged 6 hours following nanoparticle injections using an Olympus 20x UplanFl (NA 0.5) objective and the Olympus FluoView FV300 program version 4.3. Samples were excited at 488 nm with an air-cooled argon laser (Melles Griot, Carlsbad, CA) and a 748 nm infrared diode laser (Model FV10-LD748, Olympus Corporation, Tokyo, Japan). Images were collected using custom built dichroic mirrors SDM-570 and SDM-750, and emission filters BA 505–550 and BA 770 nm IF (Olympus Corporation, Tokyo, Japan). Images were acquired simultaneously on the 488 nm and 750 nm channels, and merged using OsiriX v3.2.1 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank L. Josephson, F. Reynolds, R. Kohler, E. Sun, F. Jaffer, and Y. Iwamoto for assistance, reagents, and helpful comments. This work was supported by the NIH (NHLBI U01HL080731 to S.Y.S. and R.W., NCI P50CA086355 and NCI U54CA119349 to R.W., NHLBI K08HL077186 to S.Y.S., and NIGMS P20-GM-072029 to P.A.C.), Broad Institute of Harvard and MIT (to S.Y.S., R.W., and S.L.S.), and the de Gunzburg Family Foundation at Massachusetts General Hospital (S.Y.S.).

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Figure 1.

Binding of small molecule-modified nanoparticles to macrophages (heatmap columns demarcated by orange bar) and endothelial cells (columns demarcated by green bar). The log₁₀ of bound nanoparticle concentrations (in pM) are plotted in each cell of the heatmap. Structures of four highly ranked small molecule-modified nanoparticles, along with their asymptotic permutation p-values, are shown at right. Act MP and R MP = activated and resting macrophages, respectively. Ao = aorta, IA = iliac artery, MV = microvascular, PA = pulmonary artery, SV = saphenous vein, UV = umbilical vein. FITC-CLIO = CLIO-NH₂-FITC.

Figure 2.

Relative nanoparticle binding to endothelial cells (left) or macrophages (right) for conjugates 16-3 and 16-6 (relative to binding of the starting material CLIO-NH₂-FITC (CLIO)). The concentration of 16-3 and 16-6 bound to each cell type has been normalized by dividing by the concentration of CLIO-NH₂-FITC that bound to the same cell type; binding of CLIO-NH₂-FITC has been normalized to 1 in each graph. Cell line abbreviations are the same as in Figure 1

Figure 3.

Nanoparticle localization in human carotid endarterectomy samples. The leftmost column shows nanoparticle localization (NP) *via* staining with antibodies against the FITC moiety on nanoparticles (additional 2x magnification views are shown in inserts); the middle column shows staining against CD31 (part A) or CD68 (part B); and the rightmost column shows a merged image that also includes DAPI nuclear staining. A. 16-6 and 16-3 show markedly increased (relative to CLIO-NH₂-FITC) immunofluorescence that co-localizes with CD31 signal, consistent with endothelial localization. B. CLIO-NH₂-FITC, 16-6 and 16-3 all show comparable uptake into macrophages that co-localizes with CD68 staining. L = lumen of carotid artery.

Figure 4.

In vivo intravital microscopy of 16-6 and CLIO-NH₂-FITC in mouse ear vessels following intravenous injection. A. (Left) Near-infrared channel: Angiosense-IVM 750 (red) circulates in the vascular space, and defines vessels; (Right) Merge of FITC channel (probe 16-6 localization; green) and near-infrared channel (Angiosense-IVM 750 intravascular reference; red). White arrowheads indicate regions of 16-6 localization immediately adjacent to the vessel lumen consistent with endothelial localization. B. Same as part A, but with unmodified CLIO-NH₂-FITC as the injected probe, showing that CLIO-NH₂-FITC does not accumulate adjacent to vessels. CLIO = CLIO-NH₂-FITC. Scale bar = 20 microns.

Scheme 1.

Conjugation of FITC and anhydrides to nanoparticle surfaces consisting of cross-linked, aminated dextran