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Evolving Point-of-Care Diagnostics Using Up-Converting Phosphor Bioanalytical Systems

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Up-converting phosphors promise simpler readout systems with less background at a given signal level than many other popular approaches. (To listen to a podcast about this feature, please go to the *Analytical Chemistry* website at pubs.acs.org/journal/ancham.)

Many medical diagnostic tests currently conducted in specialized testing laboratories with large and complex instrumentation are poised to move to the point of care (POC). Advances in the molecular biochemistry of diagnostic assays, coupled with more capable, better integrated, and disposable sample-handling-and-analysis cartridges provide key functional advances that enable this need-driven trend. POC assays may involve handheld analyzers, but increasingly they rely on self-contained, user-friendly tabletop systems that can be used in the emergency room; at the patient's bedside; or in a small clinic, ward, or hospital department. Such assay systems typically combine rapid (under an hour) diagnosis with less handling of the patient's sample and test results, thereby reducing time, cost, and the potential for errors.

The need for the most rapid analysis possible is clear in, for example, a blood test to measure cardiac enzymes that indicate myocardial infarct or the blood chemistry of certain critically ill patients. These applications are two early commercial examples of POC testing.[1−6] Because results can be obtained and communicated during the patient's initial visit, POC analysis also offers the following advantages: cost reductions, because samples do not have to be shipped to a central laboratory; the increased probability of patient compliance with a doctor's instructions; and lower treatment cost. Because of such improvements in medical outcome and measurable cost reductions, insurance providers and patients alike stand to benefit, potentially expanding the market for POC testing.[7,8]

Several key features are required for an integrated bioassay technology to compete with established laboratory methods. Both the technology and the biochemical assay must be highly reliable, robust, and suitable for economical mass production. The technology must be amenable to compact instrumentation and suitable for use by medical personnel at all levels, rather than requiring specially trained personnel. Multiplexed measurement of several target clinical analytes is highly desirable for increased accuracy of diagnosis or for multidisease diagnostics, such as a full analysis for all the various hepatitis viruses or for several of the common sexually transmitted diseases. In environments other than the

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traditional hospital or clinic, noninvasive biological sample collection of saliva or urine can be advantageous.

Many current POC diagnostic assays rely on fluorescence, electrochemistry, or optical absorbance in combination with movement, mixing, and binding of sample and reagents by surface-tension-driven lateral flow through porous materials such as nitrocellulose, which is by far the most common material for commercial assays. These popular detection methods and fluid-handling approaches have provided improvements in system performance, complexity, and cost. New detection approaches, such as electrochemiluminescence and magnetics, and recent developments in fluidic-sample handling and transport have also provided additional progress.

This article summarizes the principles, methods, recent experimental work, and applications of a relatively new class of particle-based "reporters" for molecular diagnostic assays, namely up-converting phosphors (UCPs). The process of up-conversion was first reported in 1966, and UCPs were first used as novel luminescent reporters in biological assays in the 1990s.[9,10] With potential to expand the range or performance of commercial POC assay technologies, UCPs promise simpler readout systems with less background at a given signal level than many other popular approaches. Over the past few years, UCP technology has begun to demonstrate its potential in human diagnostic applications.[9−15]

UCP TECHNOLOGY

UCPs are usually submicron particles, 400 nm being typical of those studied in our laboratory, made from rare-earth-doped ceramics and often coated with silica for broader bio/chemical compatibility and ease of surface functionalization. The particles have the unique property of optical up-conversion: the emission of light at a significantly shorter wavelength than that of excitation.[11] Two-photon up-conversion by energy transfer for a rare-earth ceramic doped with Yb^{3+} and Er^{3+} is shown in Figure 1a; deactivation from the $^4S_{3/2}$ state centered on Er^{3+} produces a characteristic green emission at 550 nm.[11] Figure 1b is a scanning electron micrograph of 400-nm UCP particles;[12] phosphor diameters of ≤200 nm can be synthesized if desired.[11,13]

UCP technology provides four important characteristics appropriate to POC diagnostics. First, common biological samples and matrices, as well as the materials from which assay devices are constructed, do not possess the property of optical up-conversion of the near-IR wavelengths used to excite the UCPs. For the materials discussed here, excitation energies corresponding to a range of 550 nm in the visible to ~1800 nm in the IR can populate states that emit across the full visible range;[16] the most efficient of these (800−1000 nm) yield emission from 20 to 50% of absorbed photons, depending on material composition. In contrast to conventional fluorescence-based detection, the optical background signal (sometimes referred to as the autofluorescence of the matrix or device material) is virtually zero for UCPs, a distinct advantage when detecting low concentrations of analytes.[12,13,17] Second, UCPs are photochemically stable and do not photobleach or fade.[12,16] Therefore, a phosphor-based assay can be stored indefinitely without a decrease in light-emitting efficiency, can be read by repetitive or time-integrated scans for better counting statistics, and can be archived for subsequent verification.

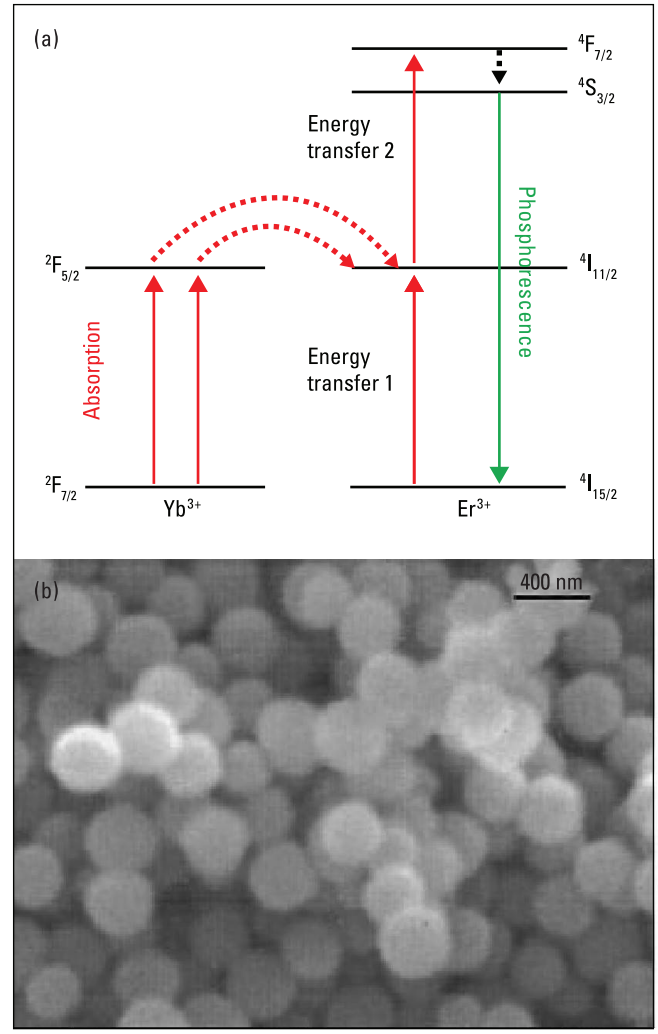

Figure 1. (a) Energy flow diagram for a Yb^{3+}−Er^{3+}-doped UCP. Illumination of Yb^{3+} raises electrons from the $^2F_{7/2}$ energy level to the $^2F_{5/2}$ level. The energy from a single IR photon is then transferred from Yb^{3+} to Er^{3+}, thereby exciting an erbium electron from the $^4I_{15/2}$ energy level to the $^4I_{11/2}$ level. An additional energy transfer from the absorption of a second IR photon further elevates the erbium electron to the higher $^4F_{7/2}$ excited state; at this point, a radiative transition spontaneously occurs to produce a photon with approximately double the energy and, therefore, also a significantly shorter wavelength. (b) Scanning electron photomicrograph of monodisperse UCP particles. (Adapted with permission from Refs. 11 and 12.)

Third, small and robust optical systems offering high sensitivity are easily constructed for use with UCP technology. The light source is usually a readily available, compact near-IR laser diode with as much as 1 W of output power.[18] The detector can be a high-sensitivity avalanche photodiode or a compact integrated photomultiplier tube (which confers greatly attenuated sensitivity at near-IR excitation wavelengths), yielding a low-cost, compact detection system.[11,19] Illumination intensities on the order of 100 W/cm^2 are easily attainable with typical 980-nm near-IR diode lasers, and compact photomultipliers can easily detect single UCP particles.[11] (Excitation spot size and exposure duration, including scanning of the laser spot, must be managed to avoid unwanted heating of the assay region at 1 W.[1]) Fourth, UCP technology can be multiplexed, either by spatial separation of the capture location for assay beads for each biochemical target

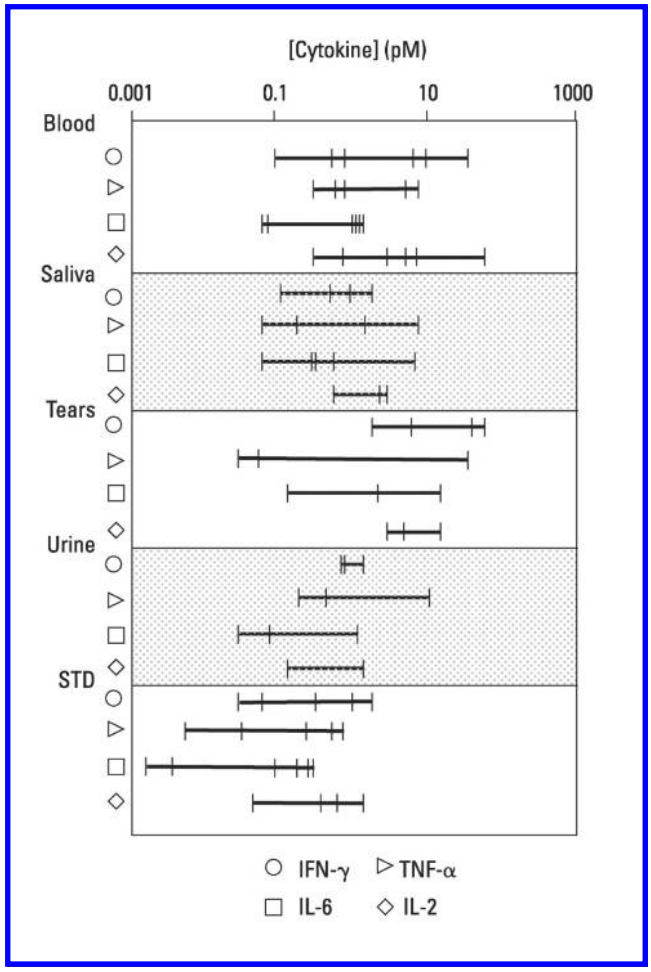

Figure 2. Survey for the most commonly assayed human cytokine proteins, illustrating each approximate detection limit from published cytokine levels in bodily fluids from healthy humans and from recombinant protein standards (STD). Various commercial kits were used for the assays.[45−89]

analyte or by the use of multiple UCP types with well-separated, narrowband emission peaks at different wavelengths. Different combinations of rare-earth emitting (Er, Ho, Tm) and absorbing (Yb, Er, Sm) ions create 20 or more unique UCP compositions that have the same absorption but different emission wavelengths;[11] thus, a unique emission color can be assigned to each of many different biomarkers in a biological sample.

UCP technology also has some manageable disadvantages. Excitation of the UCP particle requires a relatively high-power laser because the multiphoton up-conversion process has low quantum efficiency. UCP particle size, which is (thus far) quite large relative to typical proteins and nucleic acid fragments, might adversely affect specificity and the kinetics of some assays because of steric hindrance or misorientation on bead surfaces, or might limit the dynamic range of labeling when conjugated to some biomolecules.[10,15,16] Phosphors with diameters of 200 nm or less address particle size issues to some degree.[10,11,13] To date, the large particle size and low quantum efficiency have played only minor roles in the performance of bioaffinity assays, including immunochromatographic lateral flow, immunohistochemical staining, and microarray-based assays.[9,12,13,15,20]

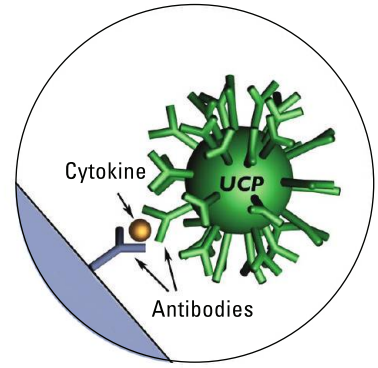

Figure 3. Selective capture of a target cytokine molecule by a surface-confined antibody, followed by attachment of a UCP particle via a second specific antibody, thereby labeling the target for optical detection.

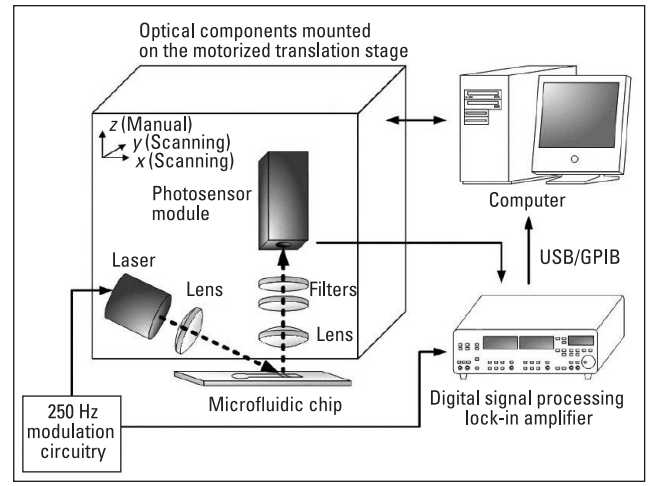

Figure 4. Block diagram of the optical scanner used to detect UCP labels in the microfluidic chip and nitrocellulose lateral-flow membrane assays for IFN-γ. The optical scanner consists of a laser excitation source, a light-collecting lens, two bandpass filters, and a photosensor module that houses a photomultiplier tube integrated with a transimpedance amplifier, all mounted on a dual-axis motorized translation stage. With a digital signal processing lock-in amplifier, this system detects fewer than 100 particles in an 8.8×10^4 μm^2 area. (Adapted with permission from Ref. 42.)

APPLICATIONS

UCP technology relies on affinity-based molecular interactions of ligands (DNA, RNA, antibodies) with analytical targets for sensitive and accurate biomarker identification and quantification. Affinity technologies such as the ELISA, which is based on molecular antigen−antibody interactions, are often used in clinical laboratories to detect cytokines, pathogens, or molecular signatures of disease. As an example of the detection limits needed for such assays, concentrations of four cytokines found in the bodily fluids of healthy humans are 0.1−100 pM, as measured by commercial affinity immunoassay technology (Figure 2 and Refs. 45−89). Cytokines are regulatory proteins involved in the body's immune response and are potentially useful as indicators of infection and stress, for pathogen classification, and for monitoring patients with autoimmune diseases.

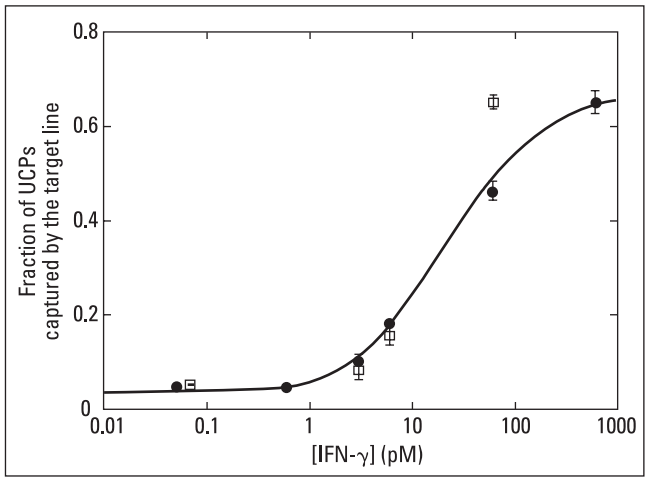

Figure 5. Detection of IFN-γ recombinant proteins on a microfluidic chip immunoassay (shaded circle) and a nitrocellulose lateral-flow immunoassay (open square). Error bars represent the standard error of the mean for three replicates. (Adapted with permission from Ref. 42.)

A typical affinity sandwich immunoassay (Figure 3) often confers greater selectivity because it uses two antibodies for the recognition of a single target protein on the assay platform, which could be a microfluidic cartridge, a lateral-flow test strip, or a microtiter plate. When a sample is added to the assay, specific antibodies preimmobilized on the platform bind and capture the target analyte. A different specific antibody conjugated to a UCP particle subsequently labels the captured target. The emitted intensity of the target-bound UCPs, which is proportional to the concentration of the analyte, is measured by an optical detection system.

UCPs have been used in lateral-flow, microarray, immunohistochemistry, and microtiter assays to detect proteins, bacteria, drugs of abuse, and nucleic acids.[9,12−15,20−22] Corstjens et al. demonstrated that UCPs enabled detection of 10^{-18} mol of nucleic acid targets without amplification of DNA in a rapid sandwich-hybridization immunochromatographic lateral-flow assay with as little as 1 μg of human DNA.[13] A sandwich assay format was also used to detect *E. coli* 0157:H7 at 10^3 org/mL in buffer or in the presence of negative-control background flora.[14] Hampl et al. demonstrated the potential of UCP technology to detect the pregnancy hormone human chorionic gonadotrophin (hCG) at concentrations as low as 10 pg/mL from a 100-μL sample with a lateral-flow assay.[12] Niedbala et al. used UCPs to detect 200 pg of the recombinant human cardiac marker troponin T with a lateral-flow assay.[23]

The protein IFN-γ was quantified with UCPs on microfluidic chips and nitrocellulose membranes. Prior to detection, UCPs were conjugated to anti-IFN-γ antibodies, and microfluidic chips (or nitrocellulose test strips) were striped with antibodies that also bind to IFN-γ (via a different epitope). The cycloolefin polymer microfluidic chips, which operate on surface-tension-driven flow, were injection-molded to contain a field of capillary-action-producing micropillars and were coated with aldehyde-functionalized dextran for covalent antibody immobilization. A 0.5-μL volume of antibodies at a concentration of 6.7 μM was dispensed onto each chip in 5-mm-long, 1-mm-wide stripes perpendicular to the direction of fluid flow.

For the assays, dilutions of 3−600 pM (50 pg/mL to 10 ng/mL) IFN-γ were prepared in HEPES buffer. UCPs at a concentration of 1 μg/mL (10^7 particles/mL) were mixed in to label the IFN-γ. A sample volume of 100 μL was applied to the microfluidic chip or nitrocellulose test strip. After a 15−20-min incubation (and an additional 20-min wash step on the microfluidic chip only), the chip or strip was scanned with a benchtop optical detector (Figure 4). IFN-γ was detected at levels as low as 50 pg/mL (3 pM) in both the nitrocellulose membrane and the microfluidic chip (Figure 5). These measurements, however, have yet to be demonstrated with biological samples.

UCP labels can help achieve detection levels comparable to those of time-resolved nanofluorescent particles, which have outstanding performance compared with other fluorescent labels.[16,19,24−29] For example, Chen et al. demonstrated that the detection limit with terbium nanoparticles in a time-resolved fluorescence assay was >100-fold lower than with the molecular dye FITC.[29] Similarly, Valanne et al. found that fluorescence assays based on Eu(III)-chelate-doped nanoparticle labels, when compared with time-resolved immunofluorometric assays, exhibited an 800-fold improvement in sensitivity with a detection limit of 5000 virus particles/mL.[19] A recent study by Ukonaho et al. found that the analytical detection limit with UCP particles was on the same order of magnitude as with Eu(III) nanoparticles.[16]

Phillips et al. developed a chip-based immunoaffinity CE assay to detect a panel of several cytokines, achieving a detection limit

Table 1. UCP performance compared with other commercially available particle bioaffinity reporters.

Analyte	Immunoassay method	Detection limit	Concentration range	Detection limit in samples from healthy humans	Ref.
IFN-γ	Fluorescent beads	7.1 pg/mL	None given	~10 pg/mL	41
	UCP	50 pg/ mL	50−800 pg/mL	None given	42
HPV-16	Colloidal gold	1 ng	None given	None given	21
	UCP	10 pg	None given	None given	21
PSA	Nanoparticles	0.76 ng/mL	None given	1−35 ng/mL	43
	Nanoparticles	0.16 ng/mL	None given	None given	16
	UCP	0.53 ng/mL	None given	None given	16
hCG	Nanoparticles	0.5 ng/mL	0.5−5 ng/mL	0.3−1.5 ng/mL	44
	UCP	10 pg/mL	None given	10 pg/mL−100ng/mL	11

Table 2. Additional biologically relevant target analytes detected by UCP technology.

Analyte	Detection limit	Ref.
Y. pestis (F1 antigen)	1 ng/mL	11
Ricin	1 ng/mL	11
B. anthracis	6×10^5 cfu/mL	11
F. tularensis	2.5×10^5 cfu/mL	11
Vaccinia	10^6 pfu	11
BG spores	1×10^4 cfu/mL	11
E. coli 0157:H7	1×10^3 org/mL	12
hCG protein	10 pg/mL	11
IFN-γ protein	50 pg/mL (3 pM)	42
Free PSA	0.53 ng/L	16

of 0.55 pg for IFN-γ with a molecular fluorophore.[30] The simultaneous detection of multiple analytes, an advantageous feature for POC diagnostics, was illustrated by the measurement of mouse IgG and ovalbumin by a lateral-flow platform with two different phosphor colors.[11,12] Table 1 summarizes published UCP performance compared with other reporter systems; Table 2 lists biologically significant analytes that have been detected with UCP technology.

A number of researchers have successfully demonstrated up-converting POC diagnostic biosensors. Cooper et al. developed a battery-operated handheld biosensor that reads lateral-flow assays primarily to detect biological warfare agents.[11] The sensor was a three-channel device capable of simultaneously detecting the emissions from three different UCPs during a single scan of the lateral-flow test strip. The handheld reader used the same detection method shown in Figure 4 with the following modifications for portability: the benchtop lock-in amplifier was substituted with a simpler integrated circuit chip; the computer interface was replaced with a modified personal digital assistant; and a miniature gear motor was used for 1D scanning of custom cartridges. Corstjens and colleagues, in conjunction with OraSure Technologies, developed a similar portable desktop reader, the Uplink reader, for on-site scanning of captured UCP-labeled biomarkers on lateral-flow test strips.[13]

TOWARD UP-CONVERTING POC DIAGNOSTICS

Often, the most desirable characteristics of POC diagnostic biosensor systems are accuracy and speed, along with the ability to integrate sample handling and measurement functions within a single consumable cartridge with low mass-production costs. The laboratory advances reported for integrated microfluidic platforms have yet to bear fruit in a large range of commercial assay systems. However, their promise for more rapid and accurate results, largely by enabling bioassays that require greater fluid- and sample-handling complexity than standard lateral-flow techniques, is becoming increasingly clear.[31−40]

Understandably, much of the recent work with UCPs has focused on integrating this technology's biosensor features with well-behaved microfluidic platforms.[42] In molecular bioassays, many of the limitations of UCP technology are the same as those of other affinity-based-reporter approaches. However, the advantages of a near-zero-background optical readout that leverages commonly available components and uses a fluor that does not bleach, fade, or wink off unexpectedly cannot be ignored. Together with detection and sample preparation methodologies that can be integrated, future UCP-based POC diagnostic systems could replace difficult laboratory assays with versatile on-site devices to monitor clinical biomarkers.

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