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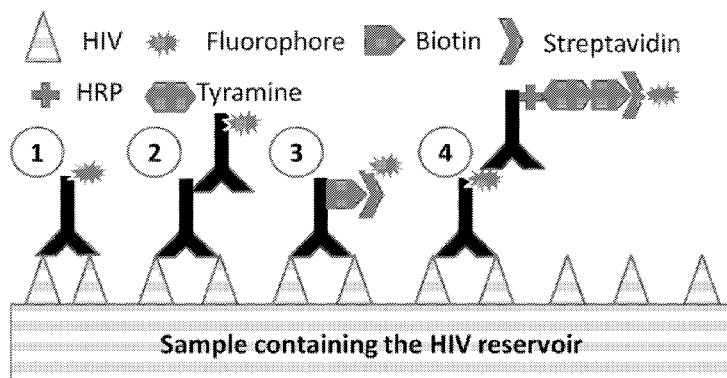


FIG. 10

(57) Abstract: A method of quantifying and detecting cells, lipids, drugs, proteins, and nucleic acids using spectral and optical de-
tection techniques. The method allows identification and/or creation of a unique signature for a given molecule based on light and
confocal microscopy, 3D reconstruction, laser imaging and/or algorithm analysis. In one embodiment, the signature corresponds to a
unique signature of the molecule examined or to dyes/antibody/probes/tags imparted therein in combination with information on
shape, localization, biogenesis, and biodistribution.

METHODS FOR THE IDENTIFICATION AND QUANTIFICATION OF PATHOGENS USING IMAGING AND MICROSCOPY TECHNIQUES

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Application No. 61/944,122
5 filed on February 25, 2014. The content of the application is incorporated herein by
reference in its entirety.

STATEMENT OF GOVERNMENT LICENSE RIGHTS

The present invention was made with government support under grant number
MH096625 awarded by the National Institute of Health. The United States government
10 may have certain rights in this invention.

FIELD OF THE INVENTION

The invention relates to a method of quantifying and detecting cells, lipids, drugs,
proteins, nucleic acids and pathogens using spectral, optical techniques and improved
detection systems (antibody and DNA/RNA probe based technology) as well as improved
15 detection devices such as high resolution cameras. More particularly, the disclosed method
allows identification and/or creation of a unique signature for a given molecule or
pathogen based on light and confocal microscopy, 3D reconstruction, laser imaging,
improved detection devices and/or algorithm analysis. In one embodiment, the signature
corresponds to a unique signature of the molecule examined or to
20 dyes/antibody/probes/tags imparted therein in combination with information on shape,
localization, biogenesis, and bio-distribution.

BACKGROUND

Many pathogens that are responsible for infectious diseases pose a diagnostic
challenge especially since the detection, quantification, and knowledge of the pathogenesis
25 are still based on quantification of infectious units, staining using different dyes, PCR, or
immune based assays.

By way of example, tuberculosis is very difficult to diagnose because it is capable
of undergoing a stage of dormancy, referred to as latency. To test tuberculosis, in the past
100 years sputum-smear and bacterial culture have been used as the tuberculosis
30 diagnostic techniques. However, sputum-smear is sub-optimal because it is incapable of
detecting *bacillum* that is not restricted to the lungs, and it cannot detect low levels or

latent TB, bacteria enclosed in granuloma, or bacterial subproducts. The tuberculin skin test (TST) is another regularly practiced clinical test that involves intradermal injection of the purified protein derivative (PPD) to diagnose tuberculosis. If there is a reaction, further confirmation is pursued through a chest x-ray and blood test. However, this test has its
5 limitations primarily due to numerous false-negatives and false-positives. Another technique is an interferon-gamma release assay (IGRA) or the TB blood test, which measure how the immune system reacts to the bacteria that cause TB bacteria by testing the person's blood in a laboratory. The technique is, however, still relatively new, costly, and it is not yet practical for worldwide application. When tested in a laboratory setting,
10 bacterial culturing is the customary practice, and depending on the media, the sensitivity is 80-90% for active TB determination. Although this sensitivity is superior to that of PCR techniques, which on average yields a 67% sensitivity rate, culturing TB is time consuming, requiring at least three weeks due to the slow growth of the bacteria.

In 2010, a more rapid, automated assay was developed at the New Jersey Medical
15 School referred to as the Xpert MTB/RIF assay. This technique has significantly advanced TB diagnosis, but latency detection still poses a great hindrance (Helb D, et al. *Journal of Clinical Microbiology* 48: 229-237 2010; Antonenka U, et al. *BMC infectious diseases* 13: 280, 2013; incorporated herein by reference in their entirety) especially for primary research of the associated pathogenesis of tuberculosis. Similar problems of detection have
20 been described for other pathogens in *in vivo* and *in vitro* conditions including HIV, Herpes viruses, hepatitis, West Nile virus, Japanese encephalitic virus and dengue as well as several others bacteria, fungus and viruses, due to latency, encapsulation, changes in metabolism, or lack of protocol for their detection.

In view of the foregoing, a solution which overcomes the above-described
25 inadequacies and shortcomings in detection of pathogens is desired. In particular, it would be desirable to develop a method of detecting a single viable pathogen *in vivo* and *in vitro* to examine latency, reactivation, and pathogenesis.

SUMMARY OF THE INVENTION

Having recognized the shortcomings of the prior art, novel methods are provided
30 that can use unique wavelength properties of pathogens, lipids, drugs, proteins, RNA, DNA and other molecular systems or targets in a sample or the unique wavelength

properties imparted by specific dyes, tags, antibody, or probes to detect and quantify any molecular system or target in a sample with unique specificity and reliability *in vivo* and *in vitro*. In one embodiment, the unique wavelength signatures of primary and secondary antibody tags are imparted to pathogens, including tuberculosis, HIV, Herpes viruses, hepatitis, West Nile virus, Japanese encephalitic virus and dengue as well as several others bacteria, fungus and viruses, as well as their distribution in individual cells are detected by confocal/STORM and light microscopy.

The greatest limitation of testing pathogens in BSL2/3 conditions is that the pathogen must be dead before it may exit the facility. Thus, to examine any pathogens, dedicated equipment, highly trained personnel, and excellent techniques of sterilization, are required to perform imaging of the “live” pathogens. Typically pathogens are fixed or inactivated so that they are no longer a hazard. Therefore, any live work must be performed within the constraints of that environment. In view of such limitation, in one embodiment, the present invention allows the detection of live pathogens such as the BSL2/3 pathogens.

Another limitation of some pathogens, especially chronic and dormant ones, is the low to undetectable expression of several markers usually used for their identification by common methodologies, such as staining and PCR. The disclosed techniques overcome such limitations by amplifying these minimal signals (*e.g.*, Proteins, RNA, DNA or unique wavelength signatures) using one or more of the techniques described below (*e.g.*, Tables 1 to 3). A better treatment of the tissues, cells or others biological samples; improved techniques of staining or identification of particular wavelength signatures, as well as improved detection systems (*e.g.*, cameras and software) allows the detection of one copy of RNA, DNA or proteins or 1 pathogen in large portions of tissue by using the large and thicker tissue sections (*e.g.*, Tables 1-3).

In one embodiment, a method of detecting molecular system or target comprises identifying a signature having unique wavelength properties of the molecular system or target; and detecting said signature by spectral and optical techniques, such as light, confocal or STORM microscopy, where the molecular system or target comprises a tissue section having a thickness between 10 and 400 μm . Such molecular system or target further comprises a pathogen and the signature having unique wavelength properties within the pathogen belongs to a lipid, a drug, a protein, a RNA or a DNA. In one

exemplary embodiment, the step of identifying the signature having unique wavelength properties of the molecular system or target is attributable to one or more antigens unique to said molecular system or target.

5 In a preferred embodiment the tissue section is hydrated and permeabilized. To eliminate background signal, the tissue section is subjected to a blocking solution and a process of endogenous biotin elimination.

10 In another embodiment, a method of detecting molecular system or target, such as a tissue section with one or more pathogens, comprises selecting a molecular system or target lacking a signature having unique wavelength properties; preparing said molecular system or target for a specific dye, tag, antibody, probe, or a combination thereof with a signature having unique wavelength properties to said molecular system or target; attaching the specific dye, tag, antibody, probe, or a combination thereof to said molecular system or target; and detecting said signature by spectral and optical techniques, where the molecular system or target comprises a tissue section having a thickness between 10 and 15 400 μm .

The present objectives, features and advantages will be apparent from the following detailed description of the invention, which is to be read in conjunction with the accompanying drawings. The scope of the invention will be pointed out in the claims. The following drawings, taken in conjunction with the subsequent description, are presented to 20 enable one of ordinary skill in the art to make and use the invention and to incorporate it in the context of particular applications.

BRIEF DESCRIPTION OF THE DRAWINGS

25 **FIG. 1A** illustrates a fluorescent microscopy image of a single viable TB bacterium in thicker tissue sections (200 μm). Rabbit was infected with *mycobacterium tuberculosis* and allowed to proceed until the infection became dormant and undetectable by PCR, CFU and dye staining. Despite the negative results of these tests, *mycobacterium tuberculosis* could be reactivated by reducing immune surveillance suggesting that the bacterium was viable but not detected. Using thicker tissue section, milder protocols of staining and antigen retrieval, the use of staining amplification as described in Table 3, 30 and improved detection systems using confocal, a single viable TB bacterium was detected in the lung of the rabbit.

FIG. 1B illustrates a fluorescent microscopy image of a single viable TB bacterium in tissue sections stained with 4',6-diamidino-2-phenylindole (DAPI). The viability of TB was identified using particular algorithms to quantify the shape and structure of the bacterium using the 3D data generated by the confocal and deconvolution analysis.

FIG. 1C is a merged image of **FIGs. 1A** and **1B**.

FIG. 2A illustrates an image of latent HIV infection of human macrophages. The virus was labeled with antibodies to HIV-p24. Actin labeled with Texas Red, was used to observe the shape of the cells, and the nuclei were probed with DAPI to quantify the total number of cells. The macrophages were infected for 28 days with no signs of active replication as determined by HIV-p24 ELISA (detection limit 0.14 pg). Using amplification systems (see tables 2 and 3) based on antibodies to HIV-p24 large accumulations of the viral protein in cells were detected in macrophages with no detectable HIV replication.

FIG. 2B illustrates an image of dengue infected cells. Dengue was stained using FITC conjugated antibodies and the signal was amplified as described in Table 3, labeled actin as a counter staining and DAPI to identify nuclei. Dengue infection was extremely low to undetectable and only 3 % of the cells became infected. Despite the low replication and numbers of infected cells, a single infected cell was detected as well as few virions inside of the infected cells.

FIGs. 3A-3F illustrate spinning disc live cell imaging of human neutrophils untreated (3A-3C) or treated with *Staphylococcus* for up to 120 hours.(3D-F), which confirms that any live cell imaging and single bacterium quantification with BSL2/3 pathogens can be performed using the disclosed techniques.

FIGs. 4A-4F are fluorescent microscopy images from six patients. **FIG. 4A:** Patient 1; **FIG. 4B:** Patient 2; **FIG. 4C:** Patient 3; **FIG. 4D:** Patient 4; **FIG. 4E:** Patient 5; **FIG. 4F:** Patient 6.

FIGs. 5A-5E are fluorescent microscopy images showing enhanced detection of extremely low concentrations of HIV proteins in viral reservoirs. **FIG. 5A:** HIV uninfected cells; **FIG. 5B:** HIV-p24 staining in uninfected cells; **FIG. 5C:** HIV latently infected cells; **FIG. 5D:** HIV-p24 staining in HIV latently infected cells; **FIG. 5E:** HIV-integrase. Bar: 30 μ m.

FIGs. 6A-6L are fluorescent microscopy images showing detection of viral components in latently infected tissue sections from three patients. **FIG. 6A**: Patient 1 DAPI staining; **FIG. 6B**: Patient 1 HIV-p24 staining; **FIG. 6C**: Patient 1 CD11b staining; **FIG. 6D**: Patient 1 merge of DAPI, HIV-p24 and CD11b staining; **FIG. 6E**: Patient 2 DAPI staining; **FIG. 6E**: Patient 2 HIV-p24 staining; **FIG. 6G**: Patient 2 CD11b staining; **FIG. 6H**: Patient 2 merge of DAPI, HIV-p24 and CD11b staining; **FIG. 6I**: Patient 3 DAPI staining; **FIG. 6J**: Patient 3 HIV-p24 staining; **FIG. 6K**: Patient 3 CD11b staining; **FIG. 6L**: Patient 3 merge of DAPI, HIV-p24 and CD11b staining. Bar: 40 μ m.

FIG. 7 shows Bacillary load in the lungs of Mtb CDC1551-infected rabbits. The values plotted are means and SD of the number of c.f.u. in the lungs of Mtb CDC1551-infected rabbits (n=3–4 rabbits per time point). No viable bacilli (i.e. 0 c.f.u.) were observed in the lungs of infected (control) rabbits at 20, 24 and 26 weeks (wk) post-infection. Starting at 20 weeks post-infection, a group of infected rabbits (n=3–4 rabbits per time point) were treated with triamcinolone for 4 weeks. The number of viable bacilli was determined in these rabbits at the end of treatment (i.e. 24 weeks post-infection) or 2 weeks after the end of treatment (i.e., 26 weeks post-infection).

FIGs. 8A-8B are microscopy images showing acid-fast staining and visualization of Mtb CDC1551 in infected rabbit lungs. **FIG. 8A** shows representative image of Mtb-infected lung sections at 4 weeks; **FIG. 8B** shows representative image of Mtb-infected lung sections at 16 weeks post-infection after staining by the ZN method and light microscopy analysis. The arrows indicate AFB; no AFB were detected in (**FIG. 8B**). Magnification, x40 (**FIG. 8A** and **FIG. 8B**); x100 (inset in **FIG. 8A**).

FIGs. 9A-9F are images of confocal imaging of Mtb CDC1551 in infected rabbit lungs after immunofluorescent staining. **FIGs. 9A-9D**: Representative images of rabbit lung sections at 8, 16, 20 and 24 weeks post-infection, respectively. **FIGs. 9E** and **9F**: Representative images of lung sections from infected rabbits treated with triamcinolone at 24 (**FIG. 9E**) and 2 (**FIG. 9F**) weeks post-infection. Arrows indicate bacilli (green) in cells (nuclei stained blue).

FIG. 10 is a schematic illustration of four different methods of immune staining. Method 1 illustrates an antibody directly conjugated to a fluorophore. This technique is commonly used in FACS analysis and also in immunohistochemistry and cytochemistry. Method 2 represents a method providing additional amplification of the signal. Method 3

demonstrates antibodies labeled with biotin and detection is by biotin-streptavidin interactions. Method 4, uses a multistep process that amplifies the number of fluorophores binding to the antigen, resulting in high sensitivity and amplification. Methods 3 and 4 are most appropriate for detection of HIV reservoirs depending on the cells and tissues being analyzed.

DETAILED DESCRIPTION OF THE INVENTION

Every pathogen, lipids, drugs, proteins, RNA, DNA and other molecular systems or targets have unique wavelength properties that can be used as a signature of such systems. Alternatively, the unique wavelength properties can be imparted to any molecular system or target that lacks such signature using specific dyes, tags, antibody, probes, or combinations thereof. In one embodiment, a novel method is described for identifying these particular wavelength signatures that can be used to detect and quantify any molecular system (or target) or pathogen with unique specificity and reliability. In another embodiment, a novel method is described for imparting one or more specific wavelength signatures to a system of interest with unique specificity and reliability. Thus, despite that the signature is minimal or not present at all, one or more critical signatures can be generated using the disclosed fluorescent techniques and amplification systems (*e.g.*, Table 3) as well as in combination with improved microscopy and detection techniques.

A critical point in the detection, in addition to the amplification of the signals, is the increased sensitivity (quantum efficiency, %), speed (frames per second, fps), and resolution (units of pixel size, μm) of a camera employed for the detection. Very dim samples are prone to quick photobleaching, or have a very short fluorescence lifetime. In particular, these samples benefit from a camera with high quantum efficiency. Normally cameras have quantum efficiencies lower than 50-65%. However, lately the detection efficiencies of cameras have substantially increased and now have a range between 60% and over 94%. Moreover, to achieve high resolution and minimal exposure, the stochastic optical reconstruction microscopy (STORM), structured illumination microscopy (SIM) and spinning disk systems generally use ultrasensitive scientific cameras (*e.g.*, iXon camera by Andor) that can provide images with a pixel size of 13-24 μm . In these cameras, capture rate reaches up to a maximal readout rate of 17 MHz with 56-11074 fps. These cameras have excellent cooling ability and several systems of noise correction

including low read noise ($<1e^-$ with EM gain). The image area pixel depth is around 180,000 e^- . This vigorous speed is uniquely capable of protecting the sample from fluorescent phototoxicity and fluorescent decay; thus, allowing extraction of images from very dim samples despite limited photon exposure. In conjunction with this increased capture rate, the software and improved image reconstruction methods of current advanced imaging systems enable resolution to molecular levels. For example, STORM technology involves a complex means of three-dimensional illumination through axial and lateral positioning, as well as superimposition through the scanning of fluorescent probes. This detection capacity of these systems including the cameras is beyond the capabilities of previous confocal microscopes that typically only detected from 250 nm x 800 nm on a xy axis. In comparison, the detection capacities of the N-SIM (Nikon Structured Illumination Microscopy) is from 85 nm x 300 nm; of the 2D Non-TIRF (Total Internal Reflection Microscopy) is from 25 nm x 800 nm; and, of the 2D TIRF is from 25 nm x 100 nm. The detection of the STORM is at a fine level with a lateral resolution of 2-30 nm and an axial resolution of 5-60 nm. This resolution can be achieved by superimposed imaging using the ratio between both an overfocused and an underfocused activated fluorophore of the specimen, called biplane axial localization microscopy. Thus, this technology can greatly surpass current confocal applications. This improved equipment as well as the improved amplification of the signal of each signature results in outstanding sensitivity for detection of pathogens, proteins, lipids, DNA or RNA.

Generally, to detect these molecules or pathogens, a novel combination of techniques and protocols are disclosed that include spectral and optical detection, 3D reconstruction, laser based imaging and/or algorithm analysis. Each technique will now be described in detail preceding by exemplary protocols of sample preparation for use in such techniques (see Tables 1-3).

Sample preparation for imaging. Samples range from tissue sections/slices (fresh, culture or fixed), cells, pathogens or others (e.g., cell extract, purified DNA, RNA, proteins; indicated as samples). Preferably, thicker tissue sections are selected that allow the detection of pathogens in extended areas. Stained or not stained samples are subjected to the following protocol with milder tissue/cell treatments for hydration, permeabilization and antigen retrieval according to the size/thickness of the tissue. Moreover, improving blocking and elimination of auto-fluorescence using low intensity lasers can reduce

particular wavelengths. Certain steps of this protocol are conditional and therefore those skilled in the art would understand that they do not have to be implemented if the condition is not met.

Generally, the protocol includes the following steps: (1) blocking endogenous
 5 biotin expression if needed; (2) blocking non-specific antibody reactivity using the blocking solution described in Table 1; (3) applying primary antibody or probe (*e.g.*, unconjugated, directly conjugated, or biotinylated). (4) applying secondary antibody or amplification system to enhance signal as described in Table 3; (5) analyzing fluorescent or individual wavelength signatures by light, confocal or STORM microscopy as
 10 described in Table 2 and 3; (6) mounting the samples using antifade reagents; (7) performing microscopy according to the protocol as described in Table 2 and 3; (8) acquiring one or more 3D images in addition to the spectrum of each analyzed molecule to detect and quantify the target desired; (9) analyzing images to identify the target in large and thicker sections or cells (see detection limits in Table 3). A summary of the exemplary
 15 techniques is presented in Table 1 to 3.

Table 1: Techniques for tissue sectioning, antigen retrieval and hydration.

	Unfixed tissue/cells	Fixed tissue/cells
Ticker tissue sections	10-400 μ m	10-400 μ m
Deparaffinize or hydration	For fresh tissue sections treat the tissue sections in a battery of alcohols to fix and permeabilize the samples by sequential alcohols. 30%-->50%-->60%-->70%-->90%-->95%-->100% twice Before staining, incubate the tissue in PBS for 20 min	For paraffin sections, use a long sequential alcohols -Deparaffinize samples -5 minutes: each solution: 30%-->50%-->60%-->70%-->90%-->95%-->100% twice. -Deparaffinize -10 minutes: xylene 100% - twice separately -Deparaffinize samples -5 minutes: each solution: 100%-twice separately → 95%--> 90% → 70%-->60%-->50%--> 30%. -Deparaffinize samples -5 minutes: each solution: 30%-->50%-->60%-->70%-->90%-->95%-->100% twice.
Tissue permeabilization	Triton 0.1 to 0.01 % in PBS	Triton 0.1 to 0.01 % in PBS
Antigen retrieval	None, SDS based antigen retrieval. 1% SDS in 0.01 M PBS (pH 7.4). -Rinse sections three times for 5 min each in PBS. -Cover sections with 1% SDS solution and incubate for 5	Required for Mycobacterium tuberculosis, Dengue, West Nile Virus, Japanese encephalitic virus, HIV, HCV, HBC and other pathogens. -Place samples for 15 minutes into boiling solution. pH 6.0, of 0.1M citric acid solution and 0.1M sodium citrate.

	minutes at room temperature. -Rinse sections three times for 5 min each in PBS. It is extremely important to wash sections well, otherwise residual SDS will denature the antibodies subsequently applied to sections.	-Samples were cooled for 20 minutes in water. For DNA and RNA. Proteinase K Solution (20 ug/ml in TE Buffer, pH 8.0): TE Buffer (50mM Tris Base, 1mM EDTA, 0.5% Triton X-100, pH 8.0). Adjust pH 8.0 using concentrated HCl (10N HCl). Store at room temperature. -Proteinase K Stock Solution in TE Buffer, pH8.0
Staining Process	Blocking solution: 0.5M EDTA, 2 % fish gelatin, 5% Ig free BSA, and horse serum (optional 3% human serum)	Blocking solution: 0.5M EDTA, 2 % fish gelatin, 5% Ig free BSA, and horse serum (optional 3% human serum)
	Essential, do not press the tissue to maintain the cyto-architecture of the tissue	Essential, do not press the tissue to maintain the cyto-architecture of the tissue
	Antibodies, probes, dyes and amplification systems are described in table 1	Antibodies, probes, dyes and amplification systems are described in table 1

In one exemplary embodiment, if the slides were stored in paraffin, they should be deparaffinized by treating the slide(s) as described in Table 1. For cells or purified pathogens, cold 70 % Ethanol or PFA 4% and subsequent triton is recommended (see Table 1). In one exemplary embodiment, the maintenance of the cyto-architecture of the tissue is critical. Thus, most of the treatments described above required extremely gentle technique of staining and imaging.

While the protocol is provided above for several preferred exemplary embodiments, the protocol can be adjusted based on the spectral/optical techniques employed and based on the needs of those skilled in the art. Table 2 provides a summary of the detection systems that can be used with the amplification systems and the disclosed microscopy technology.

Table 2: Detection system using amplification systems and microscopy technology (*only for specific protocols of detection).

Protein, second messengers, DNA or RNA-detection system	Additional amplification system	Detection system	Microscopy technique
Proteins/second messengers/organelles			
Antigen-Primary labeled antibody	None	Fluorescent/visible based	Light, confocal or STORM microscopy
Antigen-Primary-secondary	None	Fluorescent/visible	Light, confocal or

labeled antibody		based	STORM microscopy
Antigen-Primary or secondary antibodies labeled with biotin and interaction with streptavidin.	None	Fluorescent/visible based	Light, confocal or STORM microscopy
Antigen-Primary or secondary antibodies labeled with biotin and interaction with streptavidin plus TSA.	Tyramide signal amplification technology (TSA)	Fluorescent/visible based	Light, confocal or STORM microscopy
Antigen-Primary or secondary attached to beads, metals or fluorescent markers		Visible, electron microscopy or adapted fluorescent systems	Light, confocal* or STORM* microscopy
Dyes (organelle specific)	None	Fluorescent/visible based	Light, confocal or STORM microscopy
DNA and RNA			
Probes (DNA or RNA)	None	Fluorescent/visible based	Light, confocal or STORM microscopy
Probes (DNA or RNA)	Molecular Beacons and similar approaches including Bridged nuclei acid (BNA), peptide nuclei acid (PNA) and locked nuclei acid (LNA)	Fluorescent/visible based	Light, and confocal
Probes (DNA or RNA)	Tyramide signal amplification technology (TSA)	Fluorescent/visible based	Light, confocal or STORM microscopy
+ spectrum detection system			
None	None	Specific wavelengths of a protein, or drug.	Light, confocal or STORM microscopy
Antibodies/dyes/chemical changes in the target to provide a wavelength signature to a protein, DNA, or RNA that lack this characteristic	None or any described above	Fluorescent/visible based	Light, confocal or STORM microscopy
Combination of both, read at the same time the wavelength signature and an additional provided wavelength by antibodies/dyes/chemical changes	Any example described for proteins or dyes	Fluorescent/visible based	Light, confocal or STORM microscopy
Combination of both systems.	Any example described for proteins or dyes	Fluorescent/visible based	Light, confocal or STORM microscopy
+improved reading and cameras			
Improved detection and speed of cameras for low signal			
Improved cameras with low background			
Improved fluorescent and lasers to reduce specimen damage			

Improved protocols for thicker sections (up to 400 μ m) to detect pathogens in low abundance in large pieces of tissue or other biological specimens
Improved penetration protocols to deliver antibodies, dyes, and probes into biological specimens
Better and faster spectrum detection systems to identify particular signatures in the biological specimens
Faster and improved software analysis

Depending on the detection/imaging to be performed on the samples, the slides are further treated with specific solution and/or antibodies/tags. In case of fluorescence or STORM, one or more specific antibodies are used. Due to the specificity of the spectrum
5 detection system (2.5 nm of reading) several colors can be examiner at the same time, including colors in the same channels, such as GFP and FITC at the same time without slipover.

Antigen retrieval: The following method can be used for thicker tissue sections and the subsequent 3D reconstruction. The protocol is not limiting and merely provided
10 for illustrative purposes: **(1)** the slides are deparaffinized as described above; **(2)** place slides in a plastic coplin jar or Tek® staining dish and fill container with 10 mM citrate buffer, pH 6.0. *Option 1:* **(3)** place staining dish in microwave with inverted lid on top. If using a probe, set temperature to 193 °F. Different microwaves have different power, and then each one requires calibration. **(4)** mix the solution with a disposable pipet after
15 temperature has been reached and hold that temperature in the microwave for 10 min. **(5)** remove slides from microwave and set the covered dish on the counter for an additional 20 min. *Option 2:* **(3)** boil the slices in the 10 mM citrate buffer (18 ml (4.2 g Citric Acid & 20 ml ddH₂O) and 82 ml (14.7 g Sodium Citrate & 500 ml ddH₂O) adjusted to pH 6.0) for 10-12 min; **(4)** Leave the slices in PBS for 20 min to cold down. For best adherence,
20 Superfrost Plus® slides can be used (Poly-L-lysine or silane slides are also acceptable). When staining paraffin-embedded sections, antigens are affected differently by the various methods of pretreatment (citrate buffer, trypsin, or no pretreatment). Those skilled in the art would readily understand that the described protocol is merely exemplary and can be modified without diverging from the scope of the invention (*e.g.*, see Table 2).

Spectral detection: The disclosed spectral detection (or spectral detector) has the
25 ability to detect extremely narrow wavelength ranges and precise separation of emission wavelengths as small as *e.g.*, 2-20 nm, 2-15 nm, 2-10 nm, 2.5 nm, 3.0 nm, 4.0 nm, 5.0 nm, 6.0 nm, or 10 nm apart. Within the spectral detector, emission light from the biological

sample is passed through a high-efficiency grating-separating the emission light into individual components (similar to how a prism separates white light into its individual 'rainbow' components). The spectrum of light is then imaged by a precisely corrected PMT array detector (normally 32-PMT array). The individual channels on the array can be set for a specific width of emission light, as small as 2.5 nm, thus allowing the separation of exact wavelengths of light specific to the needs of the sample and/or pathogen being analyzed. This kind of spectral detector allows a spectral image to be created in a single pass, simultaneously instead of sequential, obtaining more information and reducing the exposure of the sample. The advantages of simultaneous spectral detection are increased speed of acquisition, better precision (vibration, sample movement, etc.), and the protection of biological samples from phototoxicity and photobleaching independent of the signature selected for reading. The spectrum detection can, for example, define the width of each of the 32-PMT arrays (e.g., 2-20 nm, 2-15 nm, 2-10 nm, 2.5 nm, 3.0 nm, 4.0 nm, 5.0 nm, 6.0 nm, or 10 nm) to select or identify a particular wavelength.

The disclosed spectral detection allows the separation of signals that would be otherwise impossible with wide-field fluorescence, or standard confocal microscopy. This key feature allows separation of signal from autofluorescence, fluorophores, products, drugs, or signatures that are extremely close together (e.g. GFP & Alexa488), or identification of the specific emission wavelength of an unknown fluorophore, drug, or to identify a particular signature wavelength of a pathogen. This system enables capture in 5-6 distinct colors in a sequential manner depending of the configuration of the equipment used.

Optical Detection: A key factors to detect particular signatures is the design and increased sensitivity (quantum efficiency, %), speed (frames per second, fps), and resolution (units of pixel size, μm) of an optical system or a camera. Samples that are very dim, are prone to photobleaching, or have a very short fluorescence lifetime, therefore can benefit from a camera with high quantum efficiency. Normally cameras have quantum efficiencies lower than 50-65%. However, the laser beam confocals, STORM and spinning disk systems that generally use an Andor iXon, or similar camera, can achieve the high resolution and minimal exposure with detection efficiency that ranges from 60% to over 94% and can be used for imaging having a pixel size of 13-24 μm . In these cameras, capture rate reaches up to maximal readout rate of 17 mHz with 56-11074 fps and several

systems of noise correction by excellent cooling systems, low read noise ($<1e^-$ with EM gain), and image area pixel will depth of around 180,000 e^- . This vigorous speed is uniquely capable of protecting the sample from phototoxicity and fluorescent degradation, allowing extraction of images from very dim samples despite limited photon exposure. In conjunction with this increased capture rate, the improved analysis and detection methods enable a fine level of resolution that has never been achieved before. This level of resolution allows imaging at almost molecular levels with extremely fast and reliable detection of each signature, despite dim emission of particular signatures.

For example, STORM technology involves a complex means of illumination through three-dimensional positioning axially and laterally, as well as superimposition through the scanning of fluorescent probes. This detection capacity is beyond the capabilities of regular confocals that typically detect from 250 nm x 800 nm only on an XY axis, the N-SIM (Structured Illumination Microscopy) that detects from 85 nm x 300 nm dedicated for live cell imaging, the 2D Non-TIRF (Total Internal Reflection Microscopy) localization that detects from 25 nm x 800 nm, and the 2D TIRF localization that detects from 25 nm x 100 nm. The detection of the STORM is at such a fine level with a lateral resolution of 2-30 nm and axial resolution of 5-60 nm or 50-70 nm 3D STORM resolution. This resolution can be achieved by superimposed imaging using the ratio between both an overfocused and underfocused activated fluorophore of the specimen, called biplane axial localization microscopy. Thus, the disclosed technique can surpass confocal applications, and the laser beam can also be adapted to read particular signatures in fixed and live samples by using N-SIM, spinning disk systems or similar.

3D Reconstruction: A key limitation in detection and quantification of molecular systems or targets is that it primarily provides 1D or 2D information of the 3D structure, such as PCR, staining, biochemistry, mass spectrometry, and others. Thus, most of the detection or quantification does not have a 3D or 3D resolution capability. In contrast, the disclosed 3D concept employs STORM and nSIM microscopy to reconstruct a 3D structure.

In a preferred embodiment, cyanine family fluorophores, such as Alexa 647, Cy3, Cy5, Cy5.5, and Cy7, are used. These fluorophores are capable of photoswitching to activated and de-activated states, which protects the sample from photobleaching. For example, Cy5 is able to be switched from fluorescent to dark states hundreds of times

prior to the occurrence of photobleaching. Recovery time is enhanced by the immediacy of its accompanying Cy family dye, that enables a cyanine switch due to their cyanic properties. The major notion of this is that alternative and random fluorophore activation and deactivation contrives an image of the specimen with nm localization accuracy. The random point scanning is executed to collect points on a Gaussian scale of photon detection along X, Y, and Z axes. The image that is engineered from this method is constructed at a molecular level (Bates M, et al. (2007) *Science* 317: 1749-1753; Huang B, et al. (2008) *Science* 319: 810-813; Rust MJ, et al. (2006) *Nature methods* 3: 793-795; incorporated herein by reference)

Live cell imaging: For live cell imaging, although fixed samples can also be used, the spinning disk is the standard. For superior results, however, N-SIM system is the most optimal due to the high resolution and prevention of photo damage. The spinning disc is what sets apart the N-SIM from the STORM. Spinning disc differs from regular confocal microscopes in the manner in which it focuses. Standard laser scanning microscopes focus a single beam on the specimen plane to sequentially point-scan a region of interest. Spatial filtration of the emission light is applied through a single pinhole that results in rejection of light from regions that are out of focus. However, this widely used system is extremely limited in image acquisition speed due to the photons emitted by the specimen during the pixel dwell time. In general, a regular confocal scans at the rate of 1 microsecond per pixel, thus, one image is reconstructed in around two seconds depending on the size and resolution used. In addition, most of the single-beam laser confocal microscopes produce significant damage to the cells as well as photobleaching of fluorophores due to the constant stream of photons applied to the sample during long periods of time to build an image. Depending upon the configuration, the spinning disc of this microscope is capable of reaching spinning speeds of 5,000 or 10,000 rpm, corresponding to an image capture rate of 1,000 or 2,000 frames per second, respectively. This system allows the inventor to perform long-term live-cell imaging with minimal damage to the sample analyzed. Thus, imaging of microorganisms and eukaryotic cells in real time with particular signatures and minimal damage is highly attainable. Furthermore, SIM applies the moiré effect for capturing three-dimensional images. The moiré effect is a result of moiré fringes that develop from the overlaying of different frequencies that result in multiplication of emission intensity (Gustafsson, 2005). Use of five different fluorescent colors, three

phases, and three angles converge to create a full 3D image. Combined with a piezo z stage, the movement is very rapid at 1-2 seconds per frame, using thick sections of about 5-10 μm (normally used thickness) to 20-200 μm (only using confocal adapted systems described in this application). Thus, improved systems allow adaption of several confocal techniques to the desire resolution and application.

Algorism analysis: The above described techniques can be further combined with data analysis and manipulation techniques that allow the generation and adaptation of algorithms to particular applications. The algorism analysis can be performed with Axiovision/Zen (Zeiss), CellSens (Olympus), NIS-Elements (Basic or Advance Research from Nikon), Metamorph, Volocity and/or Image-J. In a preferred embodiment, NIS-elements is used due to the flexibility and the ability to capture, display, control (peripheral), and manage data of up to 6 dimensions (X,Y, Z, lambda (wavelength), T, and multipoint). It also offers sophisticated image processing features, such as deconvolution, exclusive one-click database capability, and Extended Depth of Focus function. In particular, the software can be taught to identify particular signatures, read in 3D several samples and to identify and quantify the molecular system or target desired. If particular approaches are required, design and application of algorisms can also be included.

Integration of the new methodologies and techniques: Upon preparation of the samples according to the protocols described above to maintain antigens and structural markers, a spectrum detection system of the specimen can be performed to analyze in the sample, sample alone as well as with the specimen alone. The specimen alone can give the full spectrum of the sample without the potential contamination with subproducts of the sample. The sample can also give the spectrum without the specimen and then the specimen is analyzed in the sample to separate the wave length between both preparations. If the sample or specimen has similar wavelengths or loss their wavelength identity, a particular wavelength or an additional wavelength can be provided using dyes/antibody/probes/tags or similar detection systems. The combinations of these techniques, plus the improvement equipment and detection systems allow detection up to 1-10 photons in a 3D manner with exquisite colocalization with other 3-5 colors allowing colocalization with several other markers. Table 3 lists the exemplary embodiments of the detection systems for DNA, RNA and protein using the techniques described above. Many of the disclosed techniques are extremely reliable and require adjustment to the specific

sequence or antibody used. The detection is improved by the combination of staining, confocal, cameras, spectrum detection system and software analysis as well as specific algorithms for each application (see Table 3).

Table 3: Detection systems for DNA, RNA and protein.

Detection	Pathogen	Technique	Sensitivity	Microscopy technique
DNA	HIV-1	Probes (BNA, PNA, LNA alone or in combination with TSA)	1 copy	Light and confocal
	Herpes viruses	Probes (BNA, PNA, LNA alone or in combination with TSA)	1 copy	Light and confocal
	Other bacterial, fungus and viruses	Probes (BNA, PNA, LNA alone or in combination with TSA)	To be determined	Light and confocal
RNA	HIV-1, Nef, gag or integrase	Probes (BNA, PNA, LNA alone or in combination with TSA) or molecular beacons	1 copy for spot	Light and confocal
	JEV, NS5 and NS3	Probes (BNA, PNA, LNA alone or in combination with TSA)	1 copy for spot	Light and confocal
	WNV, NS5, NS3 and NS1	Probes (BNA, PNA, LNA alone or in combination with TSA)	1 copy for spot	Light and confocal
	Dengue1-4, NS5 and NS3	Probes (BNA, PNA, LNA alone or in combination with TSA)	1 copy for spot	Light and confocal
	Other bacterial, fungus and viruses with conserved RNA sequences	Probes (BNA, PNA, LNA alone or in combination with TSA) and molecular beacons	To be determined	Light and confocal
Proteins	HIV-1, p24, p17, gp120, tat, integrase, or nef	Any antibody based technique described in table 2 for proteins	For HIV-1 p24 detection is even in the absence of replication as determined by ELISA	Light, confocal and STORM
	JEV, NS1 and NS5	Any antibody based technique described in table 2 for proteins	Even in absence of active replication	Light and confocal
	WNV, NS1, NS3 and NS5	Any antibody based technique described in table 2 for proteins	Even in absence of active replication	Light, confocal and STORM
	Dengue1-4, CDC source	Any antibody based technique described in table 2 for proteins	Even in absence of active replication	Light, confocal and STORM
	HCV and HBV	Any antibody based technique described in table 2 for proteins	Even in absence of active replication, or negative results for	Light, confocal and STORM

			culture, CFU or PCR. 2-5 cells infected with these viruses in liver doing tickers sections and 3D reconstruction can be detected.	
	Mycobacterium Tuberculosis	Any antibody based technique described in table 2 for proteins	Even in absence of active replication, or negative results for culture, CFU or PCR. One can detect 1 bacilli in the all lung doing tickers sections and 3D reconstruction	Light, confocal and STORM
	Streptococcus and Staphylococcus	Any antibody based technique described in table 2 for proteins	Even in absence of active replication, or negative results for culture, CFU or PCR. One can detect 1 bacilli in the all lung doing tickers sections and 3D reconstruction	Light, confocal and STORM
	Other bacterial, fungus and viruses with conserved RNA sequences	Any antibody based technique described in table 2 for proteins	In Process of validation	Light, confocal and STORM
Drugs	Drugs or compounds with specific wavelengths	Spectrum detection system (no staining required)	Quantification requires calibration in each case	Confocal and STORM
Dyes	Membrane, lysosomes, mitochondrial, second messenger/indicators (calcium, pH and caged molecules),	Spectrum detection system (no staining required) in combination with excitation and emission of each one	Quantification requires calibration in each case	Confocal and STORM

Viral Detection

The methodology described herein are suitable for detection various virus. Examples of virus include HIV. Some of these cutting edge techniques most relevant to viral reservoirs include unmixing (separation of wavelengths by computer analysis not by optics that is limited) and spectrum detection (to detect extremely narrow signature wavelengths up to 2 nm), which are necessary to minimize signal background and enhance

signal to almost single molecule. These techniques are highly novel and entirely different from classical techniques of microscopy and detection.

Despite the significant success of antiretroviral treatments in controlling HIV replication, these therapies do not result in a cure. In treated individuals the virus persists in viral reservoirs whose nature and contribution to reactivation of disease are still extremely poorly understood. A critical barrier to these efforts is that most of the techniques currently used for detection and quantification of viral reservoirs (i.e. viral DNA, mRNA PCR, episomal DNA and cell activation/amplification techniques including Q-VOA) have significant limitations in terms of accuracy, precision, sensitivity, cost, long testing times, and requirement for large blood volumes from the patients (Deere, J.D. et al. (2014) PLoS One 9, e87914; Eriksson, S. et al. (2013) PLoS Pathog 9, e1003174; Graf, E.H. and O'Doherty, U. (2013) Curr Opin HIV AIDS 8, 100-5; Spina, C.A. et al. (2013) PLoS Pathog 9, e1003834; and Strain, M.C. and Richman, D.D. (2013) Curr Opin HIV AIDS 8, 106-10). In addition, these systems can detect only circulating viral reservoirs; however, large populations of latently infected cells also exist in tissues. Furthermore, all current techniques are based on detecting only one component of the viral life cycle (e.g. DNA, mRNA or proteins) (Deere, J.D. et al. (2014) PLoS One 9, e87914; Eriksson, S. et al. (2013) PLoS Pathog 9, e1003174; Spina, C.A. et al. (2013) PLoS Pathog 9, e1003834; Hilldorfer, B.B., et al (2012) Curr HIV/AIDS Rep 9, 91-100; and Rouzioux, C. and Richman, D. (2013) Curr Opin HIV AIDS 8, 170-5), resulting in several potential interpretations of the results.

As disclosed herein, new cell and tissue processing, staining and microscopy techniques were developed to obtain unprecedented levels of resolution, specificity and sensitivity to detect extremely low amounts of integrated HIV DNA, mRNA, and several HIV proteins in latently infected cells in vivo and in vitro. These methodologies are based on the latest developments in high resolution confocal microscopy, spectrum detection and unmixing systems, as well as improved techniques of signal amplification and sample processing. Using these technologies, cells from individuals were analyzed with undetectable HIV replication and were able to detect one infected cell containing one copy of HIV integrated DNA, 1-3 copies of viral mRNA and several HIV proteins expressed at extremely low levels, as well as additional cellular and molecular markers. Additionally, due to the highly flexible imaging platform, sample preparation protocols and microscopy

techniques were successfully adapted to address several of the current challenges in this area. For example, the methodologies described herein are capable of detecting viral reservoirs containing “abortive” HIV DNA sequences, incomplete mRNA expression and expression versus unspecific uptake of HIV proteins as well as their location in tissues and cells.

Historically, classical microscopy methods are not powerful enough to achieve the level of sensitivity and resolution necessary to confidently identify rare infected cells among large uninfected populations. However, recent improvements in all aspects of sample preparation, imaging and analysis have resulted in outstanding resolution and sensitivity. As shown in the examples below, these technologies were applied to successfully detect dormant *Mycobacterium tuberculosis* in latently infected individuals whose previous testing by CFU and PCR-based assays had produced negative results (Subbian, S. et al. (2014) J Med Microbiol; Rella, C.E. et al. (2014). Pathog Dis; and Eugenin, E.a.B.J.W. (2014). Methods in Virology In Press). Also, the inventor was able to detect single HIV-infected cells among millions of uninfected cells in both blood and tissues despite undetectable replication for extended periods of time. Thus, the imaging technologies disclosed herein are superior in terms of accuracy and sensitivity compared to classical techniques used to detect low levels of bacterial and viral infections. In addition, the platform disclosed herein can evaluate expression of several viral components (e.g. viral integrated DNA, mRNA and viral proteins) in a single test, significantly reducing the potential ambiguity in interpreting the results.

The imaging-based approach disclosed herein can provide a sensitive, accurate, effective, quick, and affordable clinical method to detect circulating and tissue viral reservoirs. It can be used to detect low to undetectable levels of HIV expression in animal models (SIV-infected monkeys and BLT mice) and human PBMCs and tissue sections. These studies can be designed to detect and quantify viral reservoirs in tissues by using high resolution confocal microscopy.

The techniques disclosed herein are highly innovative as it combines several improvements in sample preparation, HIV DNA/mRNA/protein staining and imaging detection systems, thus creating a unique assay to detect and quantify HIV reservoirs in resting and activated cells. Some key features of the detection system include the following: (1) High sensitivity, with identification of one copy of integrated HIV DNA

within one cell among millions (10^6 - 10^9) of negative cells, 1-3 viral mRNAs and 3-5 complexes of viral proteins per cell; (2) High accuracy, with no signal detection in uninfected samples, autofluorescence or in samples infected with other viruses; (3) High reproducibility; (4) No requirement for cell activation or HIV amplification; (5) Simultaneous use of multiple viral markers, such as viral DNA, mRNA and protein in the same sample in addition to cellular and molecular markers; (6) Cost effectiveness; (7) Time effectiveness: results can be obtained in 1 to 2 days; (8) Compatibility with a variety of samples types, including circulating cells as well as tissues; (9) Flexibility: different mutated DNA sequences can be assayed by altering probe sequences; (10) Low cell numbers/blood volumes (minimum of 10-20 ml) required for testing; (11) Adaptability to high throughput assays using a robotic improvements; (12) Combinability with several cellular/inflammatory/viral markers; and (13) Easy scalability for clinical analysis.

To provide the degree of specificity and resolution necessary for detecting and quantifying viral reservoirs, new protocols have been developed for the detection of several HIV components such as integrated HIV DNA, mRNA and HIV proteins. Some of these new and improved techniques include (1) Improved sample and tissue preparation, which allows one to conserve antigens and nucleic acids during the processing, including even archival samples; (2) Eliminating auto-fluorescence and detecting extremely narrow wavelengths by using cutting edge spectrum detection and unmixing systems; (3) Novel protocols for signal amplification for probes and antibodies as described herein; (4) Microscope automation allows one to perform fast scanning of large areas in 3 dimensions to identify the few HIV infected cells by 3D reconstructions and deconvolution. For example, in tissue analysis, one scan millions-to-billions of cells in thicker tissue sections (10 to 300 μ m) and use specialized software to identify the few cells positive for HIV DNA/mRNA and/or protein, i.e. the viral reservoirs; (5) Improved detection systems include cameras with recovery of 90% of photons per frame instead of conventional microscopy high resolution cameras that only recover around 50% of photons; (6) Improved software and computer algorithms to detect and quantify the signals generated by the different viral components.

Due to the comprehensive nature of identifying several viral components (DNA, mRNA and protein), the methodology described herein produces not merely a positive or negative result, but also the ratio of HIV DNA, mRNA and protein in each cell analyzed.

In addition to the detection of these HIV components, other cellular and molecular markers in the same cell can also be identified and depending on the platform, up to 6 markers in a single sample can be detected.

The ability to detect viral DNA, mRNA and protein in the same cell circumvents several current challenges in the field of viral reservoirs, such as detection of “abortive” viral DNA sequences that do not produce mRNA or proteins. Furthermore, some viral reservoirs do not express mRNA or proteins unless they have been reactivated. Thus, one can use the methodology described herein to examine the process of HIV reactivation: detection of three different components in the same cell should provide new insights into the dynamics of reactivation of HIV reservoirs. Described below are exemplary protocols/methods for detecting DNA and protein of virus, such as HIV.

1. Protocol of In situ hybridization to detect low copy numbers of integrated HIV-DNA

Generals:

1. Positive and negative controls

- ACH2 cells (NIH repository): positive control. This is a human lymphoid cell with only 1 integrated copy of HIV-1 DNA.
- MOLT4-IIIB: Human leukemic cell line infected with IIB strain.
- MOLT4: uninfected control

2. Sample preparation.

- Cells are fixed in 4 % paraformaldehyde (PFA) containing 0.1M sodium phosphate buffer, pH 7.4.
- Cells also can be fixed in cold 70 % Ethanol for 20 min at -20 °C.
- Formalin fixed, paraffin embedded tissue sections (fixed using 20% buffered formalin or 4% PFA)

General Method (Biotin-streptavidin qdot amplification system)

1. Sample preparation

- For cells, fix with 4% PFA containing 0.1 m sodium phosphate buffer, pH 7.4 for 60 min or RT or at 4 °C overnight. Rise the slices with PBS (3 times, three times) and dehydrate the slices in absolute ethanol and then store at -20 °C until use.
- For formalin fixed tissue (paraffin embedded). Section of 5 µm for regular microscopy or 10 to 300 µm for confocal microscopy are placed in slices. Heat

the slices in case excess of paraffin in an oven at 60 °C for 15 min and dry at 37 °C overnight. Then, Deparaffinize the section using the protocol designed in the lab (see paraffin embedded protocol and antigen retrieval protocol).

2. Antigen retrieval and pretreatment of samples

- 5 - For cells, incubate the slices in antigen retrieval solution (for 30 min) or in target retrieval solution (Dako, S1700) for 40 min at 95 °C, and allow to cool down for 20 min.
- For tissue sections, incubate the slices in antigen retrieval solution (for 30 min) or in target retrieval solution (Dako, S1700) for 40 min at 95°C, and allow to cool down for 20 min.
- 10 - For tissue sections, digest the sections with proteinase K (Dako, S3004, 2, 5 or 7 ug/ml of the enzyme depending of the thickness of the section) for 10 min at RT and then immerse the slices in 95% ethanol to kill the remaining active enzyme.
- 15 - If the tissue have endogenous biotin activity. Biotin blocking system is suggested using Biotin blocking kit (Dako, X0590). First, incubate with avidin solution for 10-30 min and then wash in TBST three times for 5 min. Second incubate with biotin solution for 10-30 min and then wash in TBST three times for 5 min.

3. Preparation of the BNA probe

- 20 - Dilute the biotin-conjugated BNA probe in hybridization solution to a final concentration of 0.5 ug/ml.
- 4. Then apply enough diluted hybridization solution (Dako, hybridization solution, PNA ISH kit, K5201) containing the BNA probe into the cells or tissue and cover with a coverslip to reduce evaporation.
- 25 5. Heat denaturation: Heat the slices at 93 °C for 5 min on a hot plate to denature the double stranded DNA.
- 6. Hybridization: Incubate the slices with the BNA probe at 45 °C for 60-90 min in a moist chamber. After incubation, immerse the slice in TBST (50 mM Tris-HCl, 300 mM NaCl, 0.1 % Tween-20, pH 7.6) to remove the cover slips.
- 30 - Wash the slices in prewarmed stringent wash solution (Dako, K5201) at 57 °C (20 min, twice)

- Incubate the slices in TBST at RT for 5 min.
- 7. Detection of the BNA probe.
 - Incubate the slices with Streptavidin-FITC, streptavidin-qdot or other dye for at least 4 hours.
- 5 - Wash the slices in TBST (5 min, three times)
- 8. In addition, one can perform IF to detect any another HIV protein or cellular marker.

After staining perform regular IF as described in the protocol for high resolution confocal in the dark. Mountain the slices with Qdot mounting media (Life sciences) or Diamond
 10 prolong gold antifate with DAPI.

Second Method (extremely high amplification using FITC-HRP-tyramine-biotin-alexa 488)

- 9. Sample preparation
 - 15 - For cells, fix with 4% PFA containing 0.1 m sodium phosphate buffer, pH 7.4 for 60 min or RT or at 4 °C overnight. Rise the slices with PBS (3 times, three times) and dehydrate the slices in absolute ethanol and then store at -20 °C until use.
 - 20 - For formalin fixed tissue (paraffin embedded). Section of 5 µm for regular microscopy or 10 to 300 µm for confocal microscopy are placed in slices. Heat the slices in case excess of paraffin in an oven at 60 °C for 15 min and dry at 37 °C overnight. Then, Deparaffinize the section using the protocol designed in the lab (see paraffin embedded protocol and antigen retrieval protocol, see notes).
- 25 10. Antigen retrieval and pretreatment of samples
 - For cells, incubate the slices in antigen retrieval solution (for 30 min) or in target retrieval solution (Dako, S1700) or solution described herein for 40 min at 95 min, and allow cooling down for 20 min.
 - 30 - For tissue sections, incubate the slices in antigen retrieval solution (for 30 min) or in target retrieval solution (Dako, S1700) or solution described herein for 40 min at 95 min, and allow cooling down for 20 min.

- Digest the sections with proteinase K (Dako, S3004, 2, 5 or 7 ug/ml of the enzyme depending of the thickness of the section) for 10 min at RT and then immerse the slices in 95% ethanol to kill the remaining active enzyme.
- If the tissue have endogenous biotin activity. Biotin blocking system is essential using Biotin blocking kit (Dako, X0590). First, incubate with avidin solution for 10-30 min and then wash in TBST three times for 5 min. Second incubate with biotin solution for 10-30 min and then wash in TBST three times for 5 min.

11. Preparation of the BNA probe

- Dilute the biotin-conjugated BNA probe in hybridization solution to a final concentration of 0.5 ug/ml.
- Then apply enough diluted hybridization solution (Dako, hybridization solution, PNA ISH kit, K5201) containing the BNA probe into the cells or tissue and cover with a coverslip.

12. Heat denaturation: Heat the slices at 93 °C for 5 min on a hot plate to denature the double stranded DNA.

13. Hybridization: Incubate the slices with the BNA probe at 45 °C for 60-90 min in a moist chamber. After incubation, immerse the slice in TBST (50 mM Tris-HCl, 300 mM NaCl, 0.1 % Tween-20, pH 7.6) to remove the cover slips.

- Wash the slices in prewarmed stringent wash solution (Dako, K5201) at 57 °C (20 min, twice)
- Incubate the slices in TBST at RT for 5 min.

14. Detection BNA probe.

- Incubate the slices with Streptavidin-FITC or other dye for 15 to 4 hours.
- Wash the slices in TBST (5 min, three times)

After staining perform regular IF as described in the protocol for high resolution confocal in the dark. Mountain the slices with Qdot mounting media (Life sciences) or Diamond prolong gold antifate solution (invitrogen)

Notes:

-For better results use Silane-coated slices.

-Some tissues have large amounts of endogenous biotin expression. Thus, after antigen retrieval applies the blocking solution.

5 -Antigen retrieval protocol

* Citrate-EDTA Antigen Retrieval Protocol

Description: Formalin or other aldehyde fixation forms protein cross-links that mask the antigenic sites in tissue specimens, thereby giving weak or false negative staining for immunohistochemical detection of certain proteins. The citrate-EDTA based solution is designed to break the protein crosslinks, therefore unmask the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections, thus enhancing staining intensity of antibodies.

Solutions and Reagents:

Citrate-EDTA Buffer (10mM Citric Acid, 2mM EDTA, 0.05% Tween 20, pH 6.2):

15 Citric acid (anhydrous) ----- 1.92 g

EDTA (Sigma, Cat# E-5134) ----- 0.74 g

Distilled water ----- 1000 ml

Mix to dissolve. Adjust pH to 6.0 and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at 4 C for longer storage. Procedure:

- 20 1. De-paraffinize sections as indicated in previous protocols
2. Pre-heat steamer or water bath with staining dish containing Citrate-EDTA buffer until temperature reaches 95-100 °C.
3. Immerse slides in the staining dish. Place the lid loosely on the staining dish and incubate for 30 minutes (optimal incubation time should be determined for every tissue section, thicker sections may require up to 50 min).
- 25 4. Turn off steamer or water bath and remove the staining dish to room temperature and allow the slides to cool for 20 minutes.
5. Rinse sections in PBS Tween 20 for 2x2 min.

30 Note: Microwave, pressure cooker or autoclave can be used as alternative heating source to replace steamer or water bath.

2. Methods to detect low levels of HIV using antibody-based technologies

Describe below are several antibody-based technologies for signal amplification to improve and detect low amounts of HIV proteins in cells, tissues, and other biological samples. The improvement in these techniques is essential to detect viral reservoirs and to design strategies to eliminate them.

The invention provides several comprehensive, integrated, and highly sensitive assays to analyze viral reservoirs by simultaneously examining integrated HIV DNA (sensitivity equal to one copy of HIV DNA per cell) or HIV mRNAs (sensitivity for few molecules) and viral proteins (sensitivity of few proteins, protocols described below). Because the detection is by imaging techniques, it does not require cell purification or amplification of the HIV components for the identification of a small number of viral reservoirs among millions of uninfected cells. This sensitivity is achieved using highly specific signal amplification systems as well as improved microscopy and optic devices (Rella, C.E. et al., (2014) *Pathog Dis* 72, 167-173 and Subbian et al., (2014) *J Med Microbiol.* 63, 1432-5. In addition to the HIV products, the methods allow one to detect several cellular/molecular markers to analyze further viral trafficking, cellular activation, compartmentalization, and HIV interacting proteins including histone acetylates, apolipoproteins, and others (up to 5-6 colors). The approach enables improved techniques of antigen recovery, staining, and confocal analysis resulting in outstanding identification and quantification of viral reservoirs. By using these methods, one can analyze millions of cells and focus only on the cells positive for viral HIV DNA/mRNA/protein using confocal microscopy, improved equipment, and imaging software. Disclosed below are two exemplary methods of detection of low levels of HIV proteins in cells. These methods can then be combined with assays for detection of HIV DNA and/or mRNA in the same samples, to obtain the most sensitive and reliable detection of viral reservoirs.

Some of the technical improvements described here include: 1) *Improved cell and tissue preparation* to conserve antigens and nuclei acids during the processing of the sample even in archival materials; 2) *The use of larger pieces of tissue or numbers of cells to analyze millions of cells* using big pinholes to generate large optical sections to detect any positive signal; 3) *The development of novel protocols enables signal amplification* for antibodies. 4) *The use of state of the art confocal systems and the automation of microscopes* allows the inventor to perform fast scanning of large areas in 3 dimensions to

identify the few HIV-infected cells by 3D reconstructions and deconvolution; 5) *The use of a spectrum detection and unmixing system* to detect extremely narrow wavelengths and eliminates auto-fluorescence; 6) *Improved detection systems* include cameras with recovery of 90% of photons per frame instead of the high resolution cameras for microscopy that only recover approximately 50% of photons and 7) Lastly, *improved software and algorithms* detect and quantify the signals generated by the different viral components.

The combination of all these factors enables the inventor to detect, quantify, and localize specific signals from HIV reservoirs.

10 **1. Materials**

1.1 Tissue sections

1. Any tissue section can be analyzed for viral reservoirs. The important point is preservation and size of the section (10-300 μm) to allow analysis of millions of cells.
- 15 2. Alcohol/Xylenes
3. Phosphate buffered saline (PBS) and Tris buffered saline (TBS)
4. Citrate
5. Fish Gelatin
6. Horse serum
- 20 7. Sudan Black
8. Sodium borohydrate
9. Pontamine sky blue and 6,6'-[(3, 3'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[4-aminobenzoic acid], tetrasodium
- 25 10. Toluidine blue
11. Triton-X
12. Biotin blocking reagents
13. Streptavidin conjugated to different fluorochromes or beads
14. Alexa conjugated secondary antibody- Goat Anti-Rabbit IgG
- 30 15. Prolong Gold anti-fade agent with DAPI

1.2. Leukocytes

1. Whole blood or leukopacks from HIV infected or uninfected individuals.

2. HIV-p24 ELISA (Perkin Elmer, Boston, MA; sensitivity: 12.5 pg/ml) or by COBAS Roche Amplicor v 1.5 (Roche, Germany; sensitivity 20 RNA copies/ml) to detect HIV infection.
3. Lysis buffer
- 5 4. Ficoll Paque plus
5. Poly-lysine glass slides
6. Phorbol myristate acetate (PMA)
7. ACH-2 and OM-10 cell lines
8. Hela cells
- 10 9. Paraformaldehyde (PFA)

2. Methods:

2.1 Equipment:

Several types of confocal microscopes can be used depending on the brand. As disclosed herein, an A1 Nikon confocal microscope with spectrum detection and unmixing separation systems was used. Using these in addition to better protocols for staining and identification of dim signals, the inventor is able to detect several latent pathogens, including low levels of HIV.

2.2 Quantification of HIV replication by ELISA or PCR:

20 Viral replication is quantified by measuring HIV-1 p24 concentrations by ELISA using a commercial kit or by PCR.

2.3. Positive and negative controls and sample fixation:

1. ACH2 (a human lymphoid) or OM-10 cells (a monocyte cell line) can be used as positive controls because each cell has only 1 integrated copy of HIV-1 DNA and produces significant amounts of viral proteins when stimulated with phorbol myristate acetate (PMA) or TNF-alpha.

2. Hela cells are used as a negative control representing uninfected cells.

3. For tissue sections, one can use human lymph nodes obtained from individuals with high or undetectable replication, as well as uninfected tissues, as positive and negative controls, respectively.

2.4 Sample preparation.

Multiple fixatives can be used for tissues, cells, or fluids including:

1. 70% cold Ethanol (-20 °C for 20 minutes)
2. Acetone
3. 20 % buffered formalin and subsequent permeabilization using 0.1 % Triton-X for 3 minutes.
- 5 4. 4 % paraformaldehyde (PFA) containing 0.1M sodium phosphate buffer, pH 7.4, and subsequent permeabilization using 0.1 % Triton-X for 3 minutes.

2.5 Deparaffining:

After fixation, and mounting into paraffin blocks, tissue sections from 10 to 300 µm are deparaffined using Ethanol-Xylene in the following order: Ethanol 30%, 50%, 10 60%, 70%, 80%, 90% and 100 %, Xylene 1 and 2 (two separate solutions), and then Ethanol 100%, 90%, 80%, 70%, 60%, 50% and 30%, and then PBS for 10 minutes. It is important to include all of the steps to assure the slow and efficient elimination of paraffin. The thickness of the section is also extremely important due to the large numbers and optical sections required for identification of viral reservoirs. Many companies and 15 facilities only prepare sections of 5-10 µm; thus, a special request to the company or training of personnel is required to obtain these types of sections.

2.6 Antigen retrieval:

There are several techniques of antigen retrieval depending on the application. A comprehensive list of antigen retrieval methods can be found at 20 www.ihcworld.com/epitope_retrieval.htm. For applications disclosed herein, the inventor used the boiling citrate buffer method for 15 minutes (pH 6.0) for thicker tissue sections (10 to 300 µm), but also obtained good results with microwave-based techniques.

2.7 Leukocyte analysis:

To analyze a significant number of circulating leukocytes, whole blood, isolated 25 PBMCs, leukopacks or specific populations of cells isolated using magnetic beads, are isolated, pelleted, and subjected to confocal analysis. The pellets can be generated directly on the glass slide or the centrifuged pellet can be fixed and cut with a cryostat. By doing this protocol, one can reduce the size and volume of cells analyzed such that millions of cells can be evaluated with a better chance of detecting viral reservoirs.

30 The following five points are critical for the detection protocols of low levels of HIV proteins using minimal amplification, because high autofluorescence can result in false positives. Most of these protocols apply to archival tissues.

2.8 Elimination of lipofuscins fluorescence:

Natural autofluorescence is due to flavins, porphyrins, and chlorophyll (mostly in plants). The main problem with these compounds present in tissues and cells is that during cutting and solvent treatments, they become redistributed, resulting in background fluorescence. However, new optical configurations to perform unmixing and spectrum detection can significantly reduce this problem. In addition, treatment of the sample with Sudan Black (0.3% in 70% Ethanol) stirred in the dark for 2 h, will reduce significantly the autofluorescence produced by lipofuscins.

2.9 Elimination of elastin and collagen autofluorescence:

This artifact is mainly found in blood vessel walls. Elastin contains several potential fluorophores when there is cross-linking of tricarboxylic amino acid with a pyridinium ring. In small vessels detection of these products is minimal, but in large vessels it is a significant problem. To eliminate autofluorescence from elastin products, incubate samples in 0.5% pontamine sky blue and 6.6'-[(3, 3'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[4-aminotetra-5-hydroxy-1,3-naphthalenedisulfonic acid], tetrasodium salt dissolved in 50 mM Tris buffer before mounting the samples. However, the use of both compounds requires extensive calibration, because pontamine sky blue fluoresces in the red channels. However, if the red channel is not to be used, it is an excellent choice.

An alternative solution is 0.1% toluidine blue for 3 minutes before mounting the samples, but this does not work in all vessels and the interpretation of the fluorescence can become complicated.

2.10 Elimination of fixative-induced fluorescence:

Aldehydes react with amines and proteins to generate fluorescent products, especially in samples incubated for a long time in fixatives. This problem occurs most often in fixatives such as glutaraldehyde and formaldehyde. For tissue sections 10 to 300 μm , incubate them 5 times for 15 minutes each in a solution of fresh borohydrate (1 mg/ml dissolved in PBS and prepared on ice). After this process, wash in PBS 3 times and discard the leftover sodium borohydrate.

2.11 Bleaching treatment:

Currently there is no company that sells appropriate light boxes, but it is relatively easy to construct. To build a specific wavelength light box (like the one used to detect

ethidium bromide in agarose gels), fluorescent tubes, especially for blue, green, red, and far red channels can be purchased from several companies. These can be used to “burn” the autofluorescence in the tissue sections before the staining process.

2.12 Endogenous biotin blocking:

5 If the tissue has endogenous biotin activity, biotin blocking is suggested using a Biotin blocking kit. First, incubate the tissue sections or pelleted leukocytes with avidin solution for 10-30 minutes and then wash in TBST 3 times for 5 minutes. Next, incubate with biotin solution for 10-30 minutes and then wash in TBST 3 times for 5 minutes.

2.14 Antibodies and Biotinylation of antibodies

10 All antibodies for staining of multiple colors will be described below. IgG biotinylation is performed using commercial Biotin labeling kits.

2.15 Data Analysis:

 Mean differences are tested by non-parametric Kruskal-Wallis analysis. If a significant F-value is obtained, means are compared with the Bofferonni-Dunn multiple
15 comparison test. A value of $p < 0.05$ is considered significant.

2.16 Multiple methods of signal amplification to detect viral reservoirs.

 Normally immune detection of proteins requires either a single antibody conjugated to a fluorescent dye or a primary antibody with a secondary antibody that amplifies the fluorescent signal. Most of the times these protocols are inefficient in
20 detecting viral reservoirs or low HIV replication in multiple systems, and only high viral replication can be detected (Orellana et al., (2014) *J Neurochem* 128, 752-63, and Hazleton et al., (2012). Purinergic receptors are required for HIV-1 infection of primary human macrophages. *J Immunol* 188, 4488-95). As described in **FIG. 10**, the combination of better protocols for fixation, antigen recovery, staining, and detection, enables exquisite
25 identification and resolution of HIV proteins, despite minimal to undetectable replication as assayed by ELISA or PCR. A critical component in optimizing these protocols is to determine the expected level of expression of HIV proteins, because most protocols described here are designed to amplify low signals (See **Note 1** below to decide the best protocol for application).

30 2.17 Staining process

 As described above, fixation, sample preparation, staining, signal amplification, and detection systems are essential for identifying and quantifying low amounts of HIV

proteins. For viral reservoirs, standard staining using directly conjugated antibodies (**FIG. 10**, method 1) and secondary conjugated antibodies (**FIG. 10**, method 2) are not sufficient to detect low levels of proteins. Thus, the methods using antibody-biotin-streptavidin-fluorophore (**FIG. 10**, method 3) and antibody-fluorophore-antibody to fluorophore conjugated to HRP-tyramine-biotin-streptavidin-fluorophore (**FIG. 10**, method 4) are highly sensitive and adaptable to determine localization, quantification, and trafficking of HIV proteins in cells, tissues, and fluids.

Approach 1

10 **Antibody conjugated to biotin-streptavidin-fluorophore amplification**

(See **FIG. 10**, 3rd method):

1. Samples are fixed, and prepared as described above (antigen retrieval, and elimination of autofluorescence) according to the sample used, tissue sections or pelleted leukocytes (go to step 4 below).
- 15 2. If tissue sections are used from paraffin blocks, heat the slides to remove excess paraffin in an oven at 60 °C for 15 minutes and dry at 37 °C overnight.
3. Deparaffinize the sections as described above using sequential alcohols and xylenes to eliminate the paraffin slowly.
4. Proceed with either antigen retrieval or blocking endogenous biotin as described above,
20 depending upon the tissue or cells being analyzed and the staining process performed. Liver, spleen, and brain are tissues with endogenous biotin.
5. Using the set up described in Figure 1, the inventor is able to detect several colors (up to 5-6 colors). The inventor can probe for nucleic acids (DAPI), HIV/SIV proteins (p24, p17, gp120, tat, integrase, or Nef), in combination with different cellular markers including
25 CD4, CD8, GFAP (an astrocyte marker), NeuN or MAP-2 (a neuronal markers) or Iba1 (a microglia/macrophage marker). For pelleted leukocytes, several cellular markers such as CD3, CD4, CD11b, CD11c, CD14, CD16, CD68, and CD163 can also be included in the same staining protocol.
6. Tissue sections or pelleted leukocytes are blocked for at least 60 minutes to overnight
30 using blocking solution (0.5M EDTA, 1% horse serum, 1% Ig free BSA, 4% human serum and 1% fish gelatin in PBS).

7. Samples are incubated overnight in primary antibody at 4 °C. A critical point is to determine how many antibodies can be used concomitantly based on antibody species and isotypes. Several combinations can be used. Some examples are:

a. HIV biotinylated antibodies (monoclonal, IgG₁)+ CD4 (rabbit antibodies)+ Iba 1 (macrophage marker)+ nuclei acid staining (DAPI).

b. HIV biotinylated antibodies (monoclonal, IgG₁) + CD4 (rabbit antibodies)+ Iba 1 (macrophage marker)+ actin staining+ nuclei acid staining (DAPI). A critical point of these experimental approaches is the determination of appropriate negative controls. For the examples described above the following controls are used:

c. Purified IgG₁ (same concentration as the HIV antibodies)+ rabbit serum or rabbit purified IgG (same concentration as the serum or of the immune IgG) and non-immune goat serum or IgG (same concentration of the Iba-1 IgGs) (See Note 2 to identify the best negative controls for experiments and antibodies).

d. Several tissues express low levels of endogenous biotin; therefore a control for this expression is required, despite inhibition of biotin binding as described above.

Importantly, negative controls using no primary antibodies or only secondary antibodies are not accurate controls. As described above, by using non-immune IgGs or serum, the inventor considers the possibility of non-specific binding to several proteins such as Fc receptors, especially in immune and inflamed tissues. All cells, tissues, and fluids to some degree have non-specific binding that is necessary to consider, especially in cases of detection of low amounts of proteins, such as found in HIV reservoirs. Thus, specificity of the antibodies must be confirmed by replacing the primary antibody with the appropriate isotype-matched control reagent, anti-IgG₁, IgG_{2A}, IgG_{2B} or the IgG fraction of normal rabbit serum depending on the primary antibody being used (see **Note 2**).

8. After incubation with the primary antibodies, at least 5 washes with PBS every 10 minutes are required to eliminate the unbound antibodies to the antigen.

9. To detect antibodies conjugated to biotin, streptavidin conjugated to a fluorophore is necessary. In addition, other streptavidin-conjugated reagents can be used such as beads or gold. Detection of low levels of HIV proteins requires at least 3 hours of incubation.

10. After incubation with the secondary antibody or conjugated streptavidin, at least 5 washes with PBS are required to eliminate the unbound streptavidin or secondary antibodies. Confocal equipment used herein has unmixing and spectrum detection systems

that enable the separation of extremely narrow wavelengths (up to 2.5 nm) to separate multiple colors without overlay.

11. After several washes to eliminate unbounded antibodies or dyes, samples are mounted using Prolong Gold anti-fade reagent with DAPI. If beads are used, using Prolong
5 Diamond anti-fade reagent with DAPI is suggested.

12. After staining, keep samples in the dark.

13. The analysis of thick samples (tissues and pelleted leukocytes) is performed first using a large pinhole just to detect HIV positive signals. After detecting the positive optical section, perform confocal microscopy in the specific XYZ positive axis with a regular
10 pinhole and good resolution to detect and quantify the localization of viral proteins using 3D reconstructions and deconvolution (see **Note 3** for details).

Approach 2

Antibody-fluorophore-antibody to fluorophore conjugated to HRP-tyramine-biotin-streptavidin-fluorophore (see **FIG. 10** 4th method, **Note 4** below for potential problems):
15 This method is essentially similar to method 1, but uses additional amplification steps.

1. Sample fixation, antigen retrieval, biotin blocking, and tissue preparation are similar to what is described for method 1 as well as described in **FIG. 10**.

2. This method includes antibodies conjugated to a fluorophore to target any HIV protein
20 in a similar manner described above. However, the main difference is that an anti-fluorophore secondary antibody conjugated to HRP (dilutions 1:600 to 1:2000) is used by adding biotinyl-tyramide for 15 minutes in the presence of 0.3 % H₂O₂ for 20 minutes to amplify the binding of the new fluorophores.

3. Wash the slides in PBS twice for 10 minutes each.

25 4. Incubate in 0.25 mg/ml streptavidin conjugated to any fluorophore for 30 minutes to 3 h in the dark.

5. Wash three times in PBS every 10 minutes in the dark

6. Incubate the slides in water for 10 minutes

7. Mount using prolong with DAPI as described above.

30

It is important to note that for all of these protocols it is essential to use pure solutions, because any contamination can be amplified and result in false positive signals (See **Note 5**).

5 **2.18 Improved microscopic analysis to detect low levels of HIV proteins in HIV reservoirs.**

10 New confocal systems, including the Nikon A1, have an improved spectral detector and unmixing systems, PMT, and cameras to improve detection and reduce potential cross contamination amount different colors. These systems allow a critical reduction in signal to noise ratio, improving the detection of specific staining. The spectral detector is the mechanism responsible for emitting light through a high-efficiency gate into its individual components, similar to how a prism separates white light into its individual ‘rainbow’ components. The improved spectral detector allows for precise separation of emission wavelengths that are then passed through a 32-photomultiplier tube (PMT) array detector that enables distinction between wavelengths as small as 2.5 nm apart. This precise detection system allows the researcher to separate and analyze specific wavelengths or eliminate autofluorescence. This technology enables the identification of signals that would be impossible to detect with wide-field fluorescence or standard confocal microscopy (Murooka et al., (2013) *J Infect Dis* 208 Suppl 2, S137-44; Paddock et al., (2014) *Methods Mol Biol* 1075, 9-47; and Shaner, (2014). *Methods Cell Biol* 123, 95-111).

25 As shown in Examples 6 and 7 below, isolated CD4⁺ T lymphocytes from individuals with undetectable HIV replication as determined by ELISA (<15 pg/ml) and PCR (<20 copies/ml) were negative for HIV-p24 staining using regular immune staining (**FIG. 5**). Using the staining and microscopy techniques described above, the few latently HIV infected cells were identified, without viral reactivation (**FIG. 6**).

30 As shown in Example 7 below, human lymph nodes were obtained from HIV infected individuals with undetectable viral replication for at least 1 year. Using signal amplification techniques described herein, the inventor was able to detect HIV proteins (**FIG. 6**) in all cases analyzed (n=9). No signal was detected in uninfected tissues. Viral reservoirs were identified not only in CD4⁺ T lymphocytes, but also in dendritic cells (CD11b positive cells, **FIG. 5**) as well as in a small population of macrophages (Iba-1

positive cells). The tissue distribution of these infected cells was donor dependent. Some donors have diffuse presence of HIV cells while others have well compartmentalized HIV infection.

Most of these techniques also can be combined with detection of HIV DNA and mRNA as described in **Note 6**. Thus, it is possible to detect HIV DNA or mRNA, viral proteins, and cellular markers at the same time in the same sample.

As disclosed herein, the inventor was able to detect several HIV proteins including HIV-p24, Nef, Vif, and integrase in one latently infected cell among 10^6 to 10^8 uninfected cells in blood smears and isolated PBMCs (pelleted preparations) from HIV infected individuals on ART with no detectable viral replication. In tissue sections obtained from HIV infected individuals with no detectable replication at the time of death, the inventor was able to detect 1.6 ± 1.2 % of T cells in lymph nodes. In brains obtained from individuals with minimal replication, 5-8% of astrocytes and 3-6 % of microglia/macrophages infected with HIV were detected.

3. Notes: The protocols described above are mostly dependent upon the quality of the starting tissue, cell separation, area of the tissue examined, and degree of viral replication. Cares should be taken at the following aspects:

1. Levels of expression and amplification: This is an essential consideration before one starts the experiment. How much staining is expected? The amplification system described in **FIG. 10**, method 4, is extremely sensitive; thus, it is not recommended to samples that are expected to have high expression of HIV proteins. In cases for which viral replication is detected by ELISA and PCR, one can use of the technique described in **FIG. 10**, method 2 or 3 instead of method 4. If the detected fluorescence is too strong, leave the stained samples at 4 °C for 1-2 weeks to allow reduction of the signal or repeat the experiment using the same samples using protocol 1 (antibody conjugated to biotin-streptavidin-fluorophore amplification, **FIG. 10**, 3rd method).

2. Negative controls: Problems with negative control antibodies, endogenous biotin, and Fc receptor expression are also common, resulting in increased background staining. If the background is uniform, changes in gain or unmixing systems in the confocal microscope may be enough to detect specific staining. If the background is non-uniform, repeat the experiment, because uneven staining is not reliable, especially by confocal microscopy. Most of the uneven staining occurs in inflamed, activated tissues

that were in formalin for long periods of time. Activated cells express high levels of Fc receptors that can bind nonspecifically to the Fc region of the immunoglobulin being used for detection (Buchwalow et al., (2011) *Sci Rep* 1, 28).

3. 3D reconstruction: Most of techniques disclosed herein involve 3D reconstructions and thicker tissue sections. Conventional microscopes are not able to detect specific signals. Analysis by confocal microscopy allows the quantification of thick tissue sections, serial optimal sections, and 3D reconstructions to quantify accurately the total numbers of HIV positive cells. Thus, the use of specific programs to perform 3D reconstruction is required such as Image J from the NIH, or others such as NIS imaging (Nikon, Japan) or Metamorph (Molecular devices, CA) (Amat et al., (2014). *Nat Methods* 11, 951-8; Ellefsen et al., (2014) *Cell Calcium* 56, 147-56; Maska et al. (2014) *Bioinformatics* 30, 1609-17; and Song et al., (2013). Three-dimensional morphometric comparison of normal and apoptotic endothelial cells based on laser scanning confocal microscopy observation. *Microsc Res Tech* 76, 1154-62).

4 Amplification problems: The tyramine amplification method is based on the binding reaction of biotinylated tyramine to phenol derivatives of a protein by peroxidase. This reaction gives nonspecific signals; therefore, it is important to pretreat specimens with methanol containing 0.3% H₂O₂ to reduce endogenous peroxidase activity.

5 Purity of the solutions: Due to the high amplification, any cross contamination between samples by using contaminated PBS will generate nonspecific signals. Thus, one should use different containers for each slide.

6 Combination of protein and other HIV markers: The described techniques could be combined with detection of HIV DNA and mRNA, resulting in a multicomponent detection system of viral reservoirs. Detection of only viral proteins is not sufficient to demonstrate viral reservoirs, because HIV proteins can be released and taken up by phagocytosis or nonspecific uptake.

Mycobacterium tuberculosis Detection

In one embodiment, the method and system described herein can be used to detect *Mycobacterium tuberculosis* in latently infected lungs.

Latent *Mycobacterium tuberculosis* (Mtb) infection (LTBI) ensues in more than 90 % of immunocompetent individuals exposed to Mtb-containing aerosols. During LTBI, the infecting bacilli presumably exist in a slow or non-replicating state. However, the

5 bacilli in LTBI cases can reactivate to cause symptomatic disease in individuals who become immunocompromised. The current understanding of the host response that drives the bacilli into latency is limited, and even less is known about the physiology of Mtb that survive in host tissues in a latent state. There is a need for detection of latent *Mycobacterium tuberculosis*.

Yet, detection of latent *Mycobacterium tuberculosis* is a challenge in the diagnosis of asymptomatic, subclinical tuberculosis. Indeed, since the enumeration of viable bacilli in infected tissue homogenates by the agar-plating method corresponds only to the number of actively replicating bacilli, this assay has limited sensitivity in detecting the total number of viable bacilli. In addition, latent bacilli are refractory to conventional microscopic detection by acid-fast staining such as the Ziehl–Neelsen (ZN) method.

As shown in the examples below, the immunofluorescence technique described herein allowed one to visualize and enumerate *M. tuberculosis* in latently infected rabbit lungs where no acid-fast-stained organisms were seen and no cultivable bacilli were obtained by the agar-plating method.

Visualization and enumeration of dormant Mtb in clinical samples can improve the sensitivity of TB diagnosis by microscopy and have implications for determining treatment outcomes (sputum AFB conversion). Identification of dormant Mtb in infected tissues can also help overcome the current inability to study the metabolism of this population of bacilli, which are difficult to detect with current methods. Finally, enumerating Mtb during LTBI would provide a useful tool for evaluating the ability of drug and vaccine candidates to eliminate such bacillary populations.

The examples set forth below also serve to provide further appreciation of the disclosed invention, but are not meant in any way to restrict the scope of the invention.

EXAMPLES

Example 1

Materials: RPMI, and DMEM medium, Fetal Bovine Serum, trypsin-EDTA, and penicillin/streptomycin were supplied by Gibco-BRL/Invitrogen (Carlsbad, CA, USA). All dyes were purchased from Invitrogen (Grand Island, NY). Antibodies were purchased from Sigma (St. Louis, MO) and Abcam (Boston, MA). For actin staining, Texas Red-X

conjugated to phalloidin (Invitrogen, Eugene, OR) was used. For nuclei probing, the ProLong Gold antifade reagent containing DAPI was added. Antibodies to GFAP, von Willebrand factor (vWF) and Cy3- or FITC-conjugated anti-rabbit IgG and Cy3 or FITC anti-mouse IgG antibodies were obtained from Sigma (St. Louis, MO). HIV-p24 monoclonal antibody was from Abcam (Cambridge, MA).

Cells: Primary cells were obtained from brain and blood. Additional experiments were also performed using Hela Cells or U87 cells (NIH AIDS Repository, Bethesda, Maryland.)

Immunofluorescence: For both tissue sections and cells: Samples were blocked, and after blocking, were stained with primary antibody for one hour at room temperature. Cells and tissues were washed to eliminate the excess of antibody, and incubated with secondary antibody conjugated to fluorophore. Samples were washed and then mounted in Prolong Gold antifade reagent and examined by confocal microscopy. *In vivo*, larger sections (30- 50 μ m) can be used with the fixed cell imaging system. Using these thicker sections optimizes the capability for antigen retrieval, as integrity of the pathogen is severely compromised during fixation.

Live Cell Imaging: Live cell imaging can be performed using any cell type. However, as an example, *Staphylococcus* was used in neutrophils focused on single cells of bacteria, 1 μ m in size. With live cell imaging, it is imperative to maintain conditions as close to physiological and/or true environmental as possible. All live cell imaging samples were maintained in constant, optimal environmental conditions as per cell line requirements, consisting of the following specifications: equilibrated carbon dioxide control; consistent humidity; double incubation system-including a thermally conserved objective to ensure immersion fluid temperature consistency, controlled specimen chamber around the apertures and stage, and a temperature controlled stage with optional coverslip and airflow control slide; an anti-vibration table in conjunction with the "Focus Drift" concept that essentially senses and re-stabilizes the objective to specimen positioning at rapid speed to prevent loss of image capture due to movement or slight temperature change. All experiments involving pathogens or primary cells are performed under protocols approved by Rutgers University for BSL2/3 approaches. For BSL2/3 pathogens the Rutgers University regulations were followed as well as the direction and

surveillance by a facility biosafety officer in assure proper organization, as well as compliance with IRB and SOP regulations.

Example 2

Using the improved staining and imaging techniques including the spectrum
5 detection system, and the specimen preparation described above, a single viable bacterium of tuberculosis in tissue sections was detected as illustrated in **FIG. 1** despite no detection of colony forming units (CFU) and acid fast staining negativity. Similarly, HIV becomes latent much like TB, hence there is a success of comorbidity and a detection difficulty as described with TB and existing diagnostic techniques. Using latently infected
10 macrophages with undetectable replication for HIV-p24 ELISA, latent HIV infection of human macrophages was detected as illustrated in **FIG. 2A** even though the replication was absent in HIVp24 ELISA. In this case, the virus was labeled with antibodies to HIV-p24 and signal was amplified using the techniques described in Table 3. Actin labeled with Texas Red, was used to observe the shape of the cells, and the nuclei were probed with
15 DAPI to quantify the total number of cells. Using these staining and improved microscopic techniques, virus (HIV-p24 and RNA) was successfully detected despite a lack of viral replication as analyzed by HIV p24 ELISA (see **FIG. 2**), as well as *in vivo* in SIV brain tissue and arteries. It is believed that the application of these techniques for HIV and tuberculosis will aid in comorbid disease detection.

20 Like tuberculosis, dengue is a historically evident pathogen. However, dengue has not received significant focus in the past fifty years until it became a more prevalent worldwide, therefore, not much research has gone into it and only recently has the CDC developed a PCR kit for detection of the different serotypes (CDC). In a similar manner to the HIV detection methodology described above, a single dengue infected cells or few to
25 single viral particles using these imaging approaches have been detected as illustrated in **FIG. 2B**. Dengue was stained using FITC conjugated antibodies, labeled actin as a counter staining, and DAPI to identify nuclei (FIG. 2B). Thus, using improved techniques and the advantages of laser based systems, cameras, and software, the inventor was able to detect down to a single pathogen in vivo and in vitro. Similar results were found for HIV, Herpes
30 viruses, hepatitis, West Nile virus, Japanese encephalitic virus and dengue as well as several others bacteria, fungus and viruses. These advancements allow the examination of latency, reactivation, and pathogenesis using sensitivity levels not possible before.

Example 3

A key limitation in the study of BSL2/3 pathogens is the ability to observe them through long term imaging which requires the recreation their physiological environment. Biosafety concerns related to dedicated equipment for these pathogens includes: device
5 contamination, cross-contamination, and complications of disinfection. Live cell imaging issues include: long term laser/photo toxicity, environmental control, selection of medium, and medium evaporation, as well as user biosafety precautions.

Many of the solutions to the typical problems of long term live cell imaging are described herein by using high resolution confocal, shorter laser exposure, better cameras,
10 algorithms, specialized software, improved spectrum detection and subsequent 3D reconstruction. The detection of pathogens in BSL2/3 conditions for *in vivo* analysis has required the generation of new protocols using thicker sections and 3D reconstruction. Moreover, pathogens can be imaged for extended periods of time-up to weeks-without significant compromise to sample integrity. In one exemplary embodiment, this technique
15 was applied by labeling *Staphylococcus aureus* and analyzing its internalization and intracellular accumulation in a time dependent manner in mammalian cells, without the loss of fluorescence and detection of phototoxicity. **FIG. 3** illustrates Nikon spinning disc live cell imaging of human neutrophils untreated (**FIG 3A-3C**) or treated with *Staphylococcus* for up to 120 hours.(**FIG. 3D-F**). In this case, the numbers of internalized
20 bacteria were quantified by fluorescence intensity and, at different time points, cells were fixed and counterstained with actin (red staining, **FIG. 3B** and **3E**) and DAPI (blue staining, **FIG. 3A** and **3D**). **FIG. 3C** corresponds to control cells without *Staphylococcus*. **FIG. 3F** corresponds to cells with internalized bacteria after 120 hours. The loss of fluorescence was minimal, 9.22%, during the time course examined. Thus, these cells after
25 five days of imaging still maintained high survival, minimal cell death, and intact fluorescence. Additional analyses that can be performed include autophagy, apoptosis, intercellular uptake or release, cell proliferation, cell death or damage, phagocytosis, and internalization. The work with *Staphylococcus* represents a proof of principal that is translatable to other bacterial/viral/fungal/parasitic forms, both current and emerging,
30 either through still or live cell imaging. These concepts serve as models to study other pathogens and finally enable detection that is sensitive enough to capture images and videos at each cellular component level.

Example 4

In this example, the methodology described herein was used for detection of HIV DNA in one cell among millions of uninfected cells. New and improved confocal microscopy protocols were developed to detect a single copy of integrated HIV DNA in one infected cell among millions of uninfected cells. Some of the techniques are based on those used in cytogenetic clinical analysis aimed at detecting specific chromosome sequences or mRNAs (Fox, C.H. et al. (1989) J Infect Dis 159, 467-71 and Webb, G.C. (2000) Methods Mol Biol 123, 29-50). The numbers of cells analyzed depended on microscope speed and software available.

Briefly, detection of HIV DNA and HIV-p24 protein was carried out in latently HIV infected CD4⁺ T lymphocytes obtained from individuals with undetectable replication. All individuals had undetectable replication by ELISA and PCR for extended periods of time (at least 2 years, >20 copies/ml or below 15 pg/ml of HIV-p24).

As shown in **FIG. 4**, a single copy of HIV integrated DNA (Arrows) was detected in non-replicating CD4⁺ T lymphocytes as determined by ELISA and PCR (>20 copies/ml). To demonstrate that these sequences of integrated DNA were productive, HIV-p24 (arrow heads) was stained. The merged picture shows the colocalization of nuclei (DAPI, blue staining to quantify total number of cells), HIV DNA (Red nuclear staining), and HIV protein (HIV-p24, green staining). It was found that not all cells with inserted DNA produced proteins. Each picture corresponds just to the positive cells in the total numbers of cells denoted on the top of each picture. Similar results were obtained in tissue reservoirs such as non-replicating macrophages, microglia and astrocytes (see below).

These techniques allowed to detect one HIV infected cell with one copy of integrated HIV DNA among millions (10^6 to 10^9 cells) of uninfected T cells using PBMCs or blood smears obtained from individuals with no detectable replication (<20 copies/ml, **FIG. 4**) as well as in un-activated ACH-2 cells. From the images shown in **FIG. 4**, it is clear that cells from some patients contained integrated HIV DNA (staining in the nucleus, arrows) but no HIV-p24 protein (**FIG. 4**, patient 1, 3 and 4), suggesting non-functional (i.e. mutated) HIV DNA sequences or may be reactivation is required. Others patients had integrated DNA but with detectable HIV-p24 production (**FIG. 4**, patient 5 and 6, arrow heads), suggesting low production of viral proteins. In addition, some individuals were negative for cells positive for HIV DNA (**FIG. 4**, patient 2). All analysis was performed

counting a total of 0.3×10^8 to 1.6×10^9 cells depending of the case analyzed. In addition, one, two or several sequences of integrated HIV DNA were detected in the host DNA by counting the signals and size of the probe.

Example 5

5 In this example, the methodology described herein was used for detection of HIV Gag mRNA using in situ hybridization.

Briefly, cultures of non-replicating latent cells were stained using BNA probes to detect HIV gag mRNA. As a control a GAPDH probe was used as a positive control for staining and to serve as a loading control (GAPDH mRNA). In these cultures no HIV
10 replication was detected by HIVp24 ELISA or by PCR (20 copies/ml). It was found that full length HIV gag and GAPDH mRNA were detected using high resolution confocal microscopy at a sensitivity of one cell in a mix of 10^6 to 10^9 uninfected cells. It was calculated that each fluorescent spot corresponded to 1 to 3 gag mRNAs. No HIV gag staining was detected in uninfected cells.

15 Example 6

In this example, the methodology described herein was used for enhanced detection of extremely low concentrations of HIV proteins in viral reservoirs. To that end, advanced optics, confocal and STORM microscopy platforms by Nikon Instruments were used.

20 Briefly, assays were performed to detect latent HIV in human CD4⁺ T lymphocytes using enhanced HIV-p24 and integrase staining. HIV-p24 staining (HIV-p24, green) or integrase (HIV-integrase, green), actin (phalloidin conjugated to Texas red, red) and nuclei (DAPI, blue) were performed.

It was found that uninfected cells did not show any HIV-p24 or integrase staining.
25 Regular protocol staining without amplification led to negative results (data not shown). Yet, the enhanced staining using the protocols described above resulted in excellent detection of HIV-p24 as well as integrase in latently infected cells with a resolution to 1-3 proteins per spot. These techniques can be combined with viral DNA and mRNA detection as shown Example 4 above.

30 Using the equipment, the inventor was able to achieve outstanding sensitivity and accuracy in detecting viral protein in latently infected cells, including CD4⁺ T lymphocytes, macrophages and CNS cells (e.g. astrocytes and microglia). As shown in

FIG. 5, isolated CD4⁺ T lymphocytes from individuals with undetectable HIV replication as determined by ELISA, PCR (<20 copies/ml) or by regular immune staining, were negative for HIV-p24 and integrase staining. Using the staining and microscopy techniques described herein, the few latently HIV infected cells that contained HIV-p24 or integrase were clearly identified. The results demonstrate that the approach can detect not only single copies of HIV DNA and mRNA (see above), but also extremely low levels of HIV proteins in latently infected cells.

Example 7

In this example, the methodology described herein was used for detection of viral components in latently infected tissue sections.

CD4⁺ T lymphocytes, dendritic cells and a population of macrophages present in human lymph nodes are viral reservoirs. Thicker lymph nodes tissue sections (200 µm) were obtained from HIV infected individuals with undetectable viral replication (for at least a year, n=9). The tissue sections were stained for nuclei (DAPI, blue), CD4, Iba-1, DC-sign, or CD11b (red staining) and HIV-p24 viral protein. Using the protocols described above, high resolution confocal microscopy and subsequent 3D reconstructions were carried out.

It was found that no staining was observed using regular immuno-fluorescence or using controls (IgG1 or pre-immune sera). Quantification of HIV infected cells was performed using the total number of cells (DAPI staining), versus the number of positive cells (CD4, Iba-1 or DC, in comparison to DAPI staining) and HIV-p24 staining (colocalization with red staining). Arrows in **FIG. 6** represent areas of positive cells. Similar results were found using DNA probes.

By using thicker tissue sections (10 to 300 µm, in contrast to 5-7 µm tissue sections used in most studies) to maintain the 3D structure of the tissue and improved techniques of tissue processing and staining, one could detect low levels of HIV DNA, mRNA and proteins, as well as several cellular and molecular markers in tissue sections from latently infected monkeys and humans (**FIG. 6**).

First, using SIV infected monkey brain tissue sections, the inventor was able to detect viral proteins in combination with apoptosis (TUNEL) and other cellular markers, such as astrocyte marker glial fibrillary acidic protein (GFAP) (Eugenin, E.A. et al. (2011) J Neurosci 31, 9456-65 and Eugenin, E.A. et al. (2011) The Journal of neuroscience : the

official journal of the Society for Neuroscience 31, 9456-65). Using high resolution confocal microscopy, astrocyte viral reservoirs (~5% of all astrocytes) were identified in SIV-infected animal with undetectable peripheral viral replication. In addition, it was found that ~15% of astrocytes in close proximity to the blood brain barrier were positive for HIV DNA (data not shown). These results were in agreement with results obtained using PCR-based HIV detection methodologies (Churchill, M.J. et al. (2009) Annals of neurology 66, 253-8).

Then, human lymph nodes were obtained from HIV infected individuals who exhibited undetectable viral replication for ≥ 2 year using clinical assays. Using signal amplification techniques described herein, the inventor was able to detect HIV proteins (**FIG. 6**) and DNA (data not shown) in all cases analyzed (n=9). No signal was detected in uninfected tissues (data not shown). Viral reservoirs were identified not only in CD4⁺ T lymphocytes (data not shown), but also in dendritic cells (CD11b positive cells, red staining, Fig. 4) as well as a small population of macrophages (Iba-1 positive cells, data not shown). The tissue distribution of these infected cells was donor dependent (data from 3 different patients are shown below). Some donors showed diffuse presence of HIV infected cells while others contained extremely clustered HIV infection in few cells.

As demonstrated in Examples 4-7 above, the method described herein is highly sensitive.

Integrated HIV DNA: Assays were carried out to analyze blood smears or purified PBMCs, brain sections, and lymph nodes obtained from HIV infected individuals undergoing effective ART with no detectable viral replication. In blood smears/PBMCs HIV DNA positive cell was detected at 1 in 10^6 - 10^9 uninfected cells as demonstrated in the examples section (n=15). In brain sections, using 3D reconstruction, it was found that 5.1 $\pm$ 3.2 to 11.98 $\pm$ 8.77 % of cells (astrocytes and microglia/macrophages, respectively) were positive for HIV DNA (n=25). In lymph nodes, 2.1 $\pm$ 1.67 % of HIV DNA positive cells were detected, including CD4⁺ T lymphocytes, dendritic and macrophages (n=22).

HIV mRNA: Using probes for HIV-gag mRNA, it was found that most of the circulating HIV DNA positive cells were negative for gag mRNA. If all the data of all patients analyzed were combine, only 2.3 $\pm$ 1.87% of cells with HIV DNA also contained HIV-gag mRNA in unactivated PBMCs obtained from HIV infected individuals with

undetectable replication (n=15). In individuals with low but detectable HIV replication (20-30 copies/ml) only 12 ± 6.56 % of the HIV DNA positive cells are also positive for gag mRNA (n=15).

HIV protein: Several HIV and SIV proteins were detected, including HIV-p24, HIV-p17, SIV-p27, Nef, Vif and integrase, in up to 1.88 ± 1.03 % of the cells also positive for HIV DNA in no reactivated cells. In addition, several positive cells for HIV-p24 and integrase with no HIV DNA were detected suggesting that several cells in the circulation can take up viral proteins without replication. It was hypothesize that these HIV-p24 and integrase positive cells are positive by taking up proteins from tissue reservoirs that express these proteins. The data in tissue sections indicated that one can detect several viral markers (HIV DNA, mRNA and proteins) in particular population of cells such as CD4, Iba-1, DC-SIGN, CD11b and GFAP positive cells depending of the tissue analyzed.

These results demonstrate that the method and system allow one to (i) detect more than one HIV component simultaneously, enabling unprecedented levels of sensitivity of detection and quantification of viral reservoirs, even without reactivation; (ii) detect one HIV infected cell within millions of uninfected cells in blood as well as in tissue sections; (iii) correlate the presence of viral reservoirs with several cellular markers and other proteins involved in silencing or reactivation of viral reservoirs. This capability is essential to understanding the formation and potential elimination of viral reservoirs in circulation as well as in tissues. The method and system were highly reliable, inexpensive, sensitive, and applicable to clinical trials.

Example 8

In this example, the methodology described herein was used for detection of *Mycobacterium tuberculosis* in latently infected lungs of a rabbit model of pulmonary tuberculosis (Subbian et al., Journal of Medical Microbiology (2014), 63, 1432–1435).

METHODS

Rabbit infection and lung bacterial load determination.

Mtb CDC1551 were grown in Middlebrook 7H9 medium (Difco) and used to infect New Zealand white rabbits through a 'snout-only aerosolexposure system', as described previously (Subbian et al., 2011, PLoS Pathog 7, e1002262). Four rabbits were killed at 3 h post-exposure (T50); the lungs were homogenized in sterile saline and serial dilutions were placed on Middlebrook 7H11 agar plates (Difco) and incubated for 4–5

weeks to determine the initial bacillary load by the agar plating method, as described previously (Subbian et al., 2011, Open Biol 1, 110016.). At 8, 16, 20, 24 and 26 weeks post-infection, lung homogenates were prepared from groups of three or four rabbits and used for further analysis. To reactivate LTBI, groups of rabbits (n=3–4 rabbits per time point) were immunosuppressed with triamcinolone (Kenalog) treatment at 16 mg kg⁻¹, administered through intramuscular injection for 4 weeks, starting at 20 weeks postinfection, when the animals had fully cleared the infection from the lungs (0 c.f.u. observed on agar plates) (Flynn et al., 2008 Experimental animal models of tuberculosis. In Handbook of Tuberculosis, pp. 389–426. Edited by S. H. E. Kaufmann & E. Rubin. Weinheim: Wiley-VCH Verlag.; Subbian et al., 2012). Lungs from triamcinolone-treated and -untreated rabbits were harvested for the agar plating method and histological and immunohistological staining. The limit of detection for the assay was ,10 bacilli. All rabbit procedures were approved by the Institutional Animal Care and Use Committee of Rutgers Biomedical and Health Sciences.

Histology.

Formalin-fixed lung sections from Mtb CDC1551-infected rabbits (n=3–4 rabbits per time point) were paraffin embedded, cut into 5 mm sections and stained using the ZN method to visualize bacilli, as described previously (Kaplan et al., 2003, Infect Immun 71,7099–710; Subbian et al., 2011, Open Biol 1, 110016). At least three stained lung sections from each rabbit per time point were analyzed by a trained pathologist. Images were photographed using a Nikon Microphot-FX microscope.

Immunofluorescence staining of Mtb in rabbit lung tissue sections.

Paraffin-embedded rabbit lung tissue infected with Mtb CDC1551 was cut into 30–300 mm sections. Sections were dehydrated in a stepwise manner by passing them through an alcohol gradient in the following order: 30, 50, 60, 70, 90, 95, 100 and 100 %, and two passes in xylene for 5 min each. The sections were then rehydrated in the reverse order, followed by 15 min incubation in sterile 16 PBS. To improve the permeability of the antibodies, tissue sections were incubated in 0.1 % Triton X-100 for 1 min. The sections were boiled in citrate buffer (pH 6.0) for 20 min to retrieve Mtb antigens. Unlike standard 5 µm sections used for histological staining, thicker tissue sections allowed the inventor to examine extensive X, Y and Z optical planes by confocal microscopy. This particular feature allowed the inventor to identify infected host cell types and their

location, as well as numbers of bacilli. Non-specific blocking was performed by incubating the sections in blocking solution containing 0.5 M EDTA, 1 % fish gelatin, 1 % IgG-free BSA and 1 % each of horse and human serum. Tissue sections were incubated overnight at 4 °C with a primary anti- Mtb–biotin antibody (GeneTex). This antibody was produced using Mtb purified protein derivative (a mixture of soluble proteins secreted by Mtb) and reacts with the Mtb antigens lipoarabinomannan, ESAT-6, CFP-10, 38 kDa protein, antigen 16 (HspX), Hsp65 (GroEL) and MoeX, as demonstrated by Western blot analysis (GeneTex).

Following incubation with the primary antibody, slides were washed three times with sterile 16 PBS and incubated with streptavidin conjugated to FITC (Invitrogen) at 1 : 1000 dilution for 3 hours at room temperature, and again washed three times in sterile 16 PBS. Sections were mounted on glass slides using ProLong Gold Antifade reagent with 49,6-diamidino-2-phenylindole (Invitrogen) and examined using an A1 confocal microscope equipped with a spectrum detection system (Nikon). Antibody specificity was confirmed by replacing the primary antibody with a non-specific myeloma antigen of the same isotype or with a non-immune serum. To assure objective quantification of Mtb in the stained sections, analysis was performed in a blinded manner. At least two stained lung sections from each rabbit per time point (n=3–4 rabbits per time point) were stained and analyzed.

RESULTS

The agar plating method revealed a mean of 3.29 log₁₀ Mtb CDC1551 implanted in the rabbit lungs at T=0. At 8 and 16 weeks post-infection, the bacillary loads were 4.2 and 1.9 log₁₀ c.f.u., respectively. No bacilli (i.e., 0 c.f.u.) were observed in the rabbit lungs at 20 weeks post-infection (**FIG. 7**). Importantly, immunosuppression of LTBI rabbits by triamcinolone treatment for 4 weeks, starting at 20 weeks post-infection, facilitated resumed bacillary growth to about 3.4 and 4.4 log₁₀ c.f.u. at 24 and 26 weeks post-infection, respectively. At these time points, no viable bacilli were observed in the untreated, Mtb CDC1551-infected (control) rabbit lungs (**FIG. 7**). Thus, during LTBI, the infecting bacilli remained viable in a non-replicating state that was unable to form colonies on agar plates. Consistent with these results, acid-fast bacilli (AFB) were seen in ZN-stained lung sections at 4 weeks post-infection (**FIG. A**). However, no AFB were seen in lung sections after 16 weeks post-infection (**FIG. 8B**).

To determine whether non-cultivable and ZN stain-negative bacilli could be detected in the lungs, immunofluorescent anti-Mtb antibody-stained sections were analyzed in three dimensions for bacilli size, length and fluorescent signature evaluation using NIS-Elements software (Nikon) (**FIG. 9**). First, it was found that the three-dimensional size, length and medium fluorescence intensity of a full-body bacillus were 124 967±29 754 pixels (arbitrary units) for 145 bacilli analyzed. This approach was used to scan the Mtb CDC1551-infected lung sections from three rabbits per time point and recorded the pixel values from 32 fields in multiple X-Y-Z sections (**FIG. 9**, Table 4). Small clumps of bacilli were seen in the lung sections at 8 weeks post-infection. Thereafter, individual bacilli were seen scattered in association with single or small clusters of host cell nuclei. Immunosuppression resulted in the reappearance of small clumps of bacilli (**FIG. 9**). Consistent with enumeration by the agar plating method, more bacteria were observed by immunofluorescent staining confocal microscopy at 8 weeks compared with 16 and 20 weeks, and in lung sections from immunosuppressed rabbits. Importantly, means of about 2.35, 2.27 and 2.2 log₁₀ bacilli were found in the lungs of infected rabbits at 20, 24 and 26 weeks post-infection, respectively, when 0 c.f.u. was observed on agar plates (Table 4).

Table 4. Quantification of fluorescence intensity and calibration of corresponding number of bacilli in Mtb CDC1551 infected rabbit lung sections

Time post-infection	Pixels (arbitrary units)		Number of bacteria*	
	Mean	SD	Mean	SD
8 weeks	797 324.16	168 333.33	6.38	0.87
16 weeks	374 820.59	51 093.07	4.59	0.41
20 weeks	191 863.28	24 384.57	1.54	0.19
24 weeks: no treatment	158 243.41	13 699.53	1.27	0.11
26 weeks: no treatment	143 522.97	15 288.84	1.15	0.12
24 weeks: immunosuppressed	3 609 472.74	737 211.79	18.44	28.53
26 weeks: immunosuppressed	1 490 651.68	166 659.92	11.92	20.85

*Calculated based on 32 X-Y-Z fields using 1.24 ± 0.46 cm tissue sections.

In this study, it was determined, using immunofluorescencebased confocal microscopy imaging, the presence of intact Mtb that did not form colonies in the conventional agarplating method and were not AFB-positive by ZN staining in the lungs of latently infected rabbits.

It will be appreciated by persons skilled in the art that the present invention is not limited to what has been particularly shown and described. Rather, the scope of the present invention is defined by the claims which follow. It should further be understood that the above description is only representative of illustrative examples of embodiments. The
5 description has not attempted to exhaustively enumerate all possible variations. The alternate embodiments may not have been presented for a specific portion of the invention, and may result from a different combination of described portions, or that other undescribed alternate embodiments may be available for a portion, is not to be considered a disclaimer of those alternate embodiments. It will be appreciated that many of those un-
10 described embodiments are within the literal scope of the following claims, and others are equivalent. Furthermore, all references, publications, U.S. Patents, and U.S. Patent Application Publications cited throughout this specification are hereby incorporated by reference as if fully set forth in this specification.

CLAIMS

1. A method of detecting a molecular system in a sample, comprising identifying a signature having unique wavelength properties of the molecular system; and

5 detecting the signature by spectral, optical and specific amplification techniques .

2. The method of claim 1, wherein the step of identifying comprises identifying one or more antigens unique to said molecular system.

10 3. The method of claim 1, wherein the step of identifying comprises hydrating, and permeabilizing the sample.

4. The method of claim 4, wherein the step of identifying further comprises eliminating background signal by using a blocking solution.

15 5. The method of claim 4, wherein the step of identifying further comprises eliminating endogenous biotin.

20 6. The method of claim 5, wherein the step of identifying further comprises eliminating one or more wavelengths using low power lasers before the staining process.

7. A method of detecting a molecular system in a sample, comprising selecting a molecular system lacking a signature having unique wavelength properties;

25 preparing said molecular system for a specific dye, tag, antibody, probe, or a combination thereof with a signature having unique wavelength properties to said molecular system;

attaching the specific dye, tag, antibody, probe, or a combination thereof to said molecular system; and

30 detecting said signature by spectral and optical techniques.

8. The method of any one of claims 1-7, wherein the sample comprises a tissue section.

9. The method of any one of claims 1-8, wherein the molecular system further
5 comprises a pathogen.

10. The method of claim 9, wherein the signature within the pathogen belongs to a lipid, a drug, a protein, RNA or a DNA.

10 11. The method of claim 7, wherein the specific dye, tag, antibody, or probe is selected from Table 2.

12. The method of any one of claims 1-11, wherein the molecular system, the pathogen, and the spectral and optical techniques are prepared as described in Table 3.

15 13. The method of any one of claims 1-12, wherein the spectral and optical techniques have low ratio of signal to background.

14. The method of any one of claims 1-13, wherein the spectral and optical
20 techniques are carried out via a camera having high sensitivity for detecting low abundance pathogens.

15. The method of any one of claims 1-14, wherein the spectral and optical techniques further comprise microscopy and 3D analysis.

25 16. The method of any one of claims 1-15, wherein the spectral and optical techniques are selected from the group consisting of light, confocal and STORM microscopy.

30 17. The method of claim 7, wherein the step of preparing comprises blocking endogenous biotin expression if needed; and blocking non-specific antibody reactivity using the blocking solution described in Table 1.

18. The method of claim 17, wherein the step of attaching comprises applying a primary antibody or probe selected from unconjugated, directly conjugated, or biotinylated; and applying a secondary antibody or amplification system to enhance signal as described in Table 3.

19. The method of claim 18, wherein the step of detecting comprises analyzing fluorescent or individual wavelength signatures by light, confocal or STORM microscopy as described in Tables 2 and 3; mounting the samples using antifade reagents; performing microscopy according to the protocol as described in Table 2 and 3; acquiring one or more 3D images in addition to the spectrum of each analyzed molecule to detect and quantify a target desired; and analyzing images to identify the target in large and thicker sections or cells.

20. The method of claim 8, wherein the section has a thickness between about 10 and 400 μm .

21. The method of claim 9, 10, 12, or 14, wherein the pathogen is selected from the group consisting of HIV, dengue virus, West Nile virus, Japanese encephalitis virus, and *Mycobacterium tuberculosis*.

22. The method of any one of claims 1-21, wherein the detecting step comprises separating one or more wavelength ranges from the molecular system, and detecting the signature in one or more of the wavelength ranges, wherein each wavelength range is about 2-20 nm wide or apart.

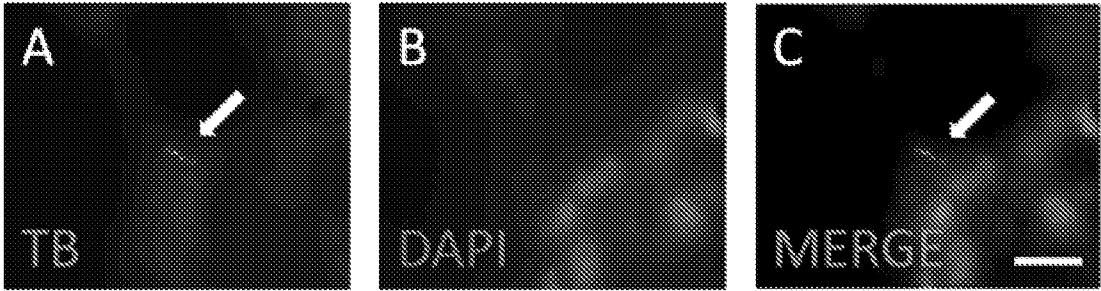


FIG. 1

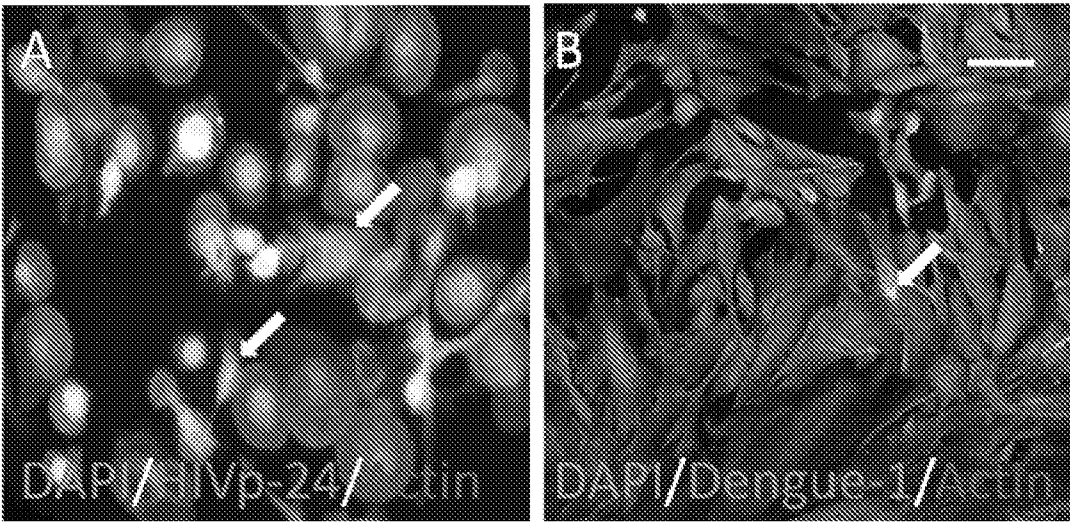


FIG. 2

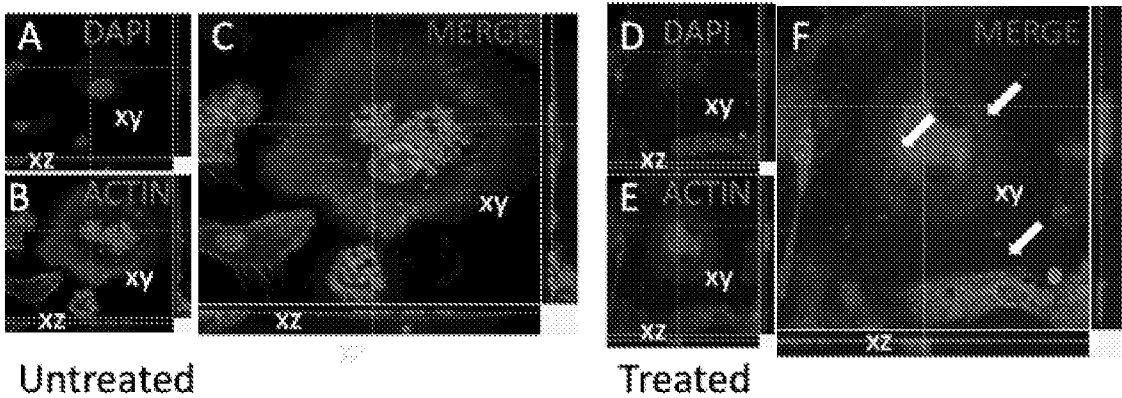


FIG. 3

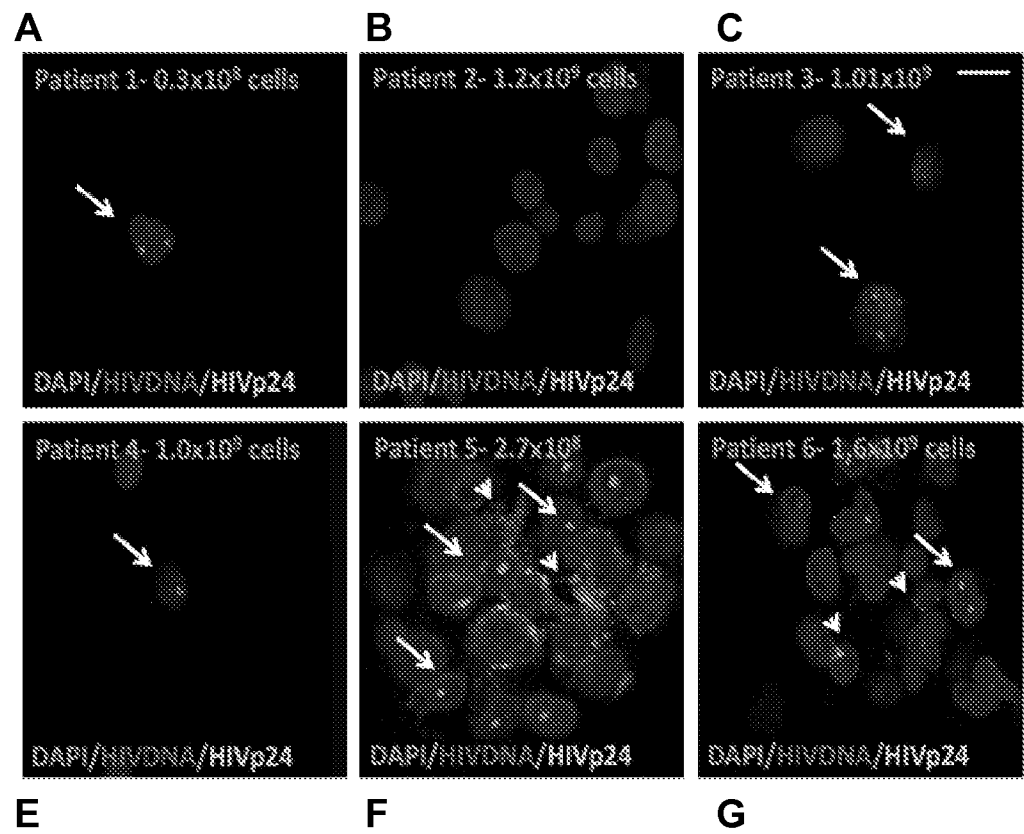


FIG. 4

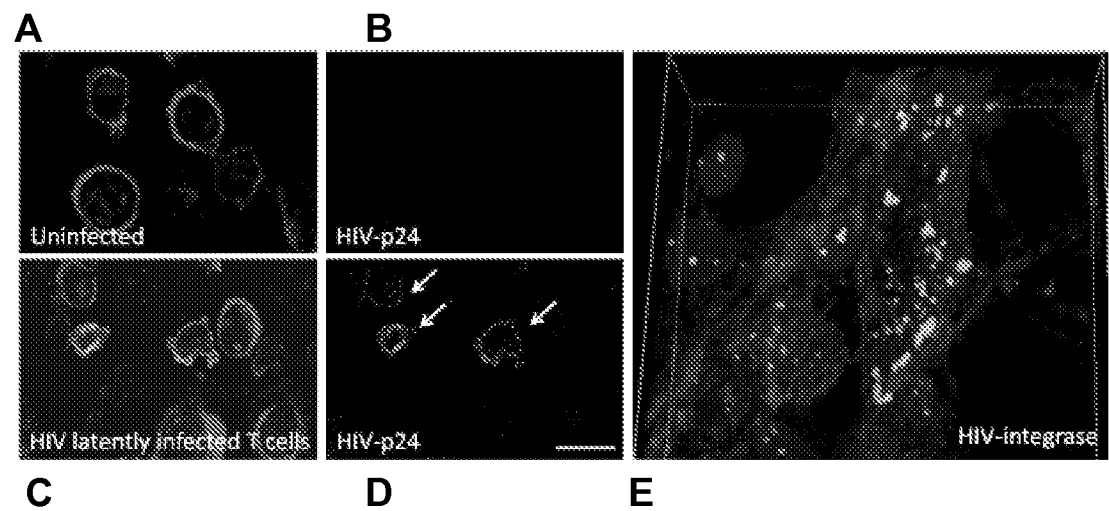


FIG. 5

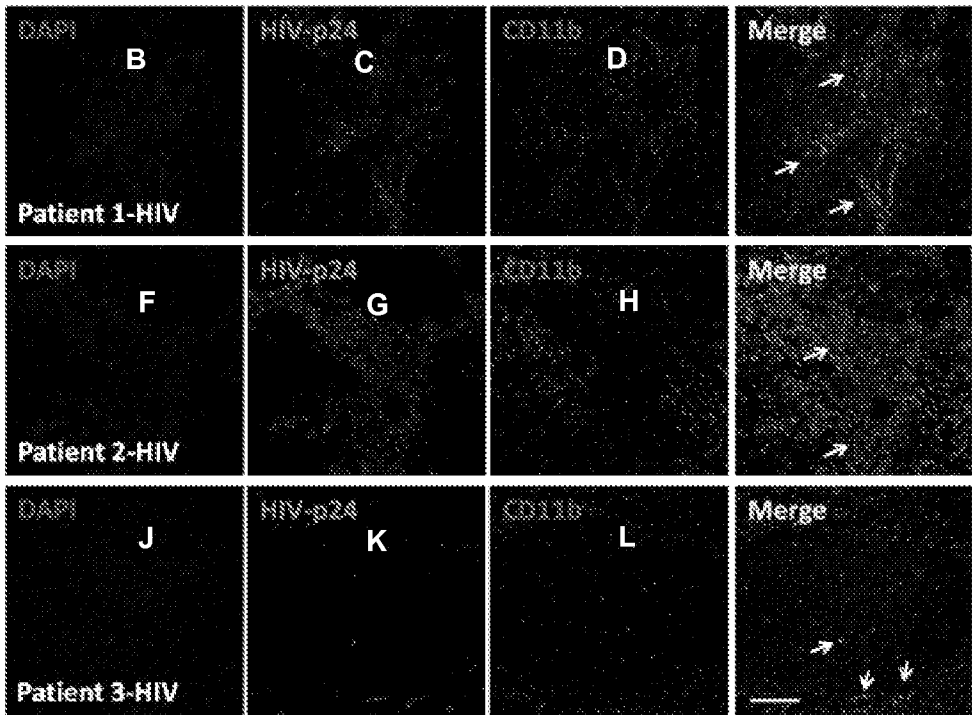


FIG. 6

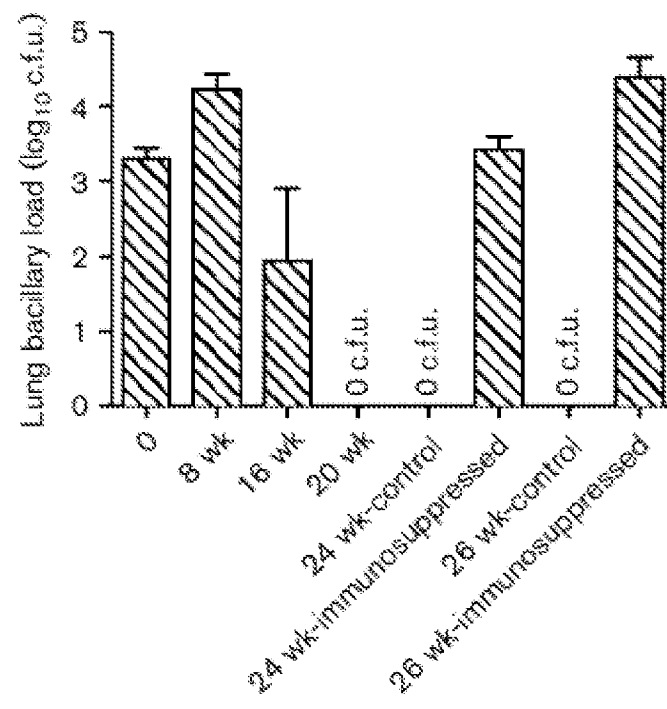


FIG. 7

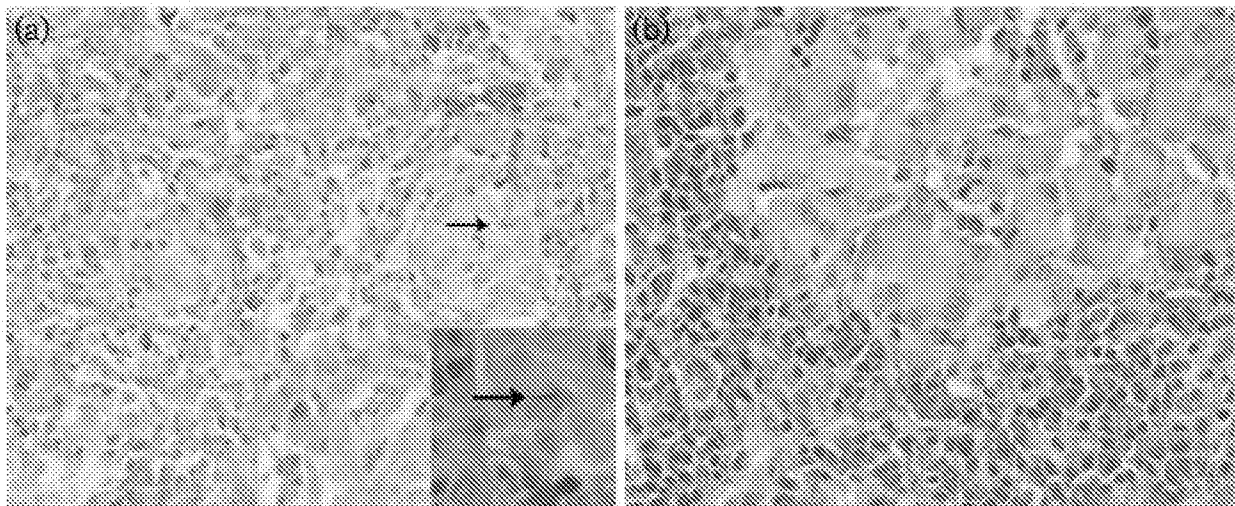


FIG. 8

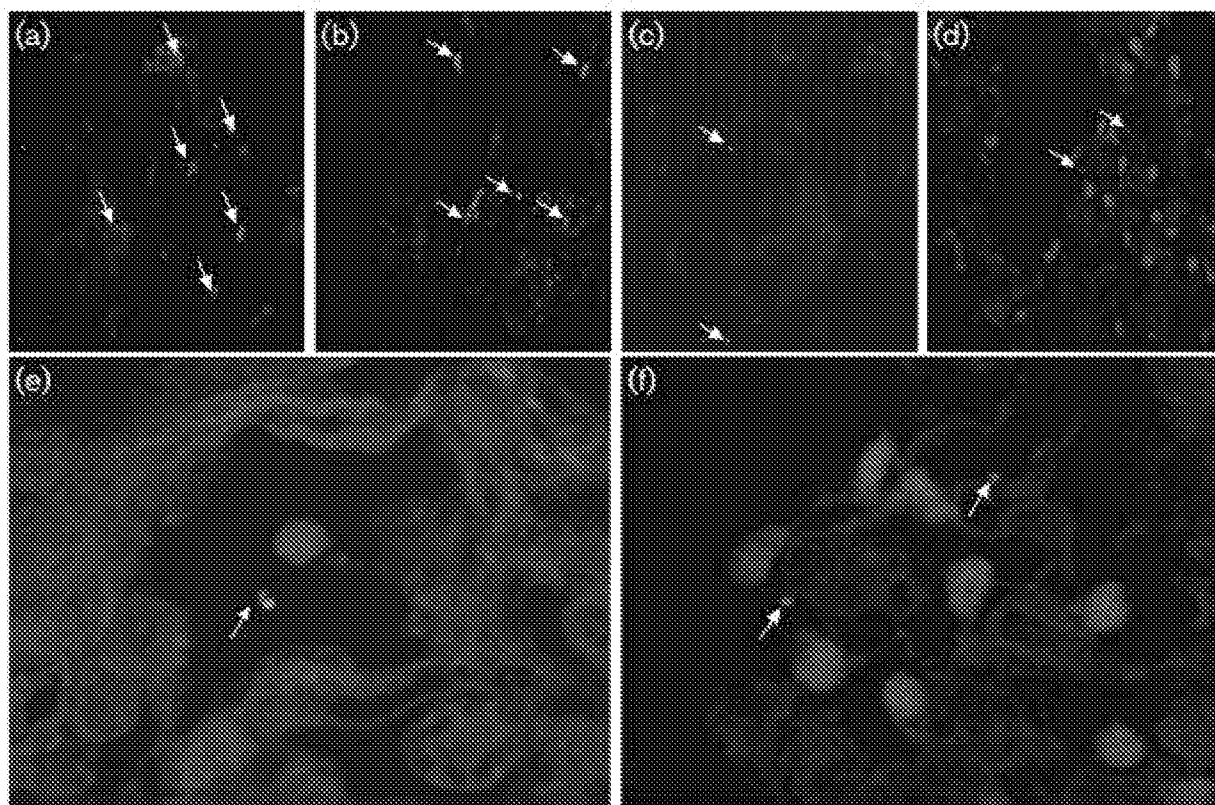


FIG. 9

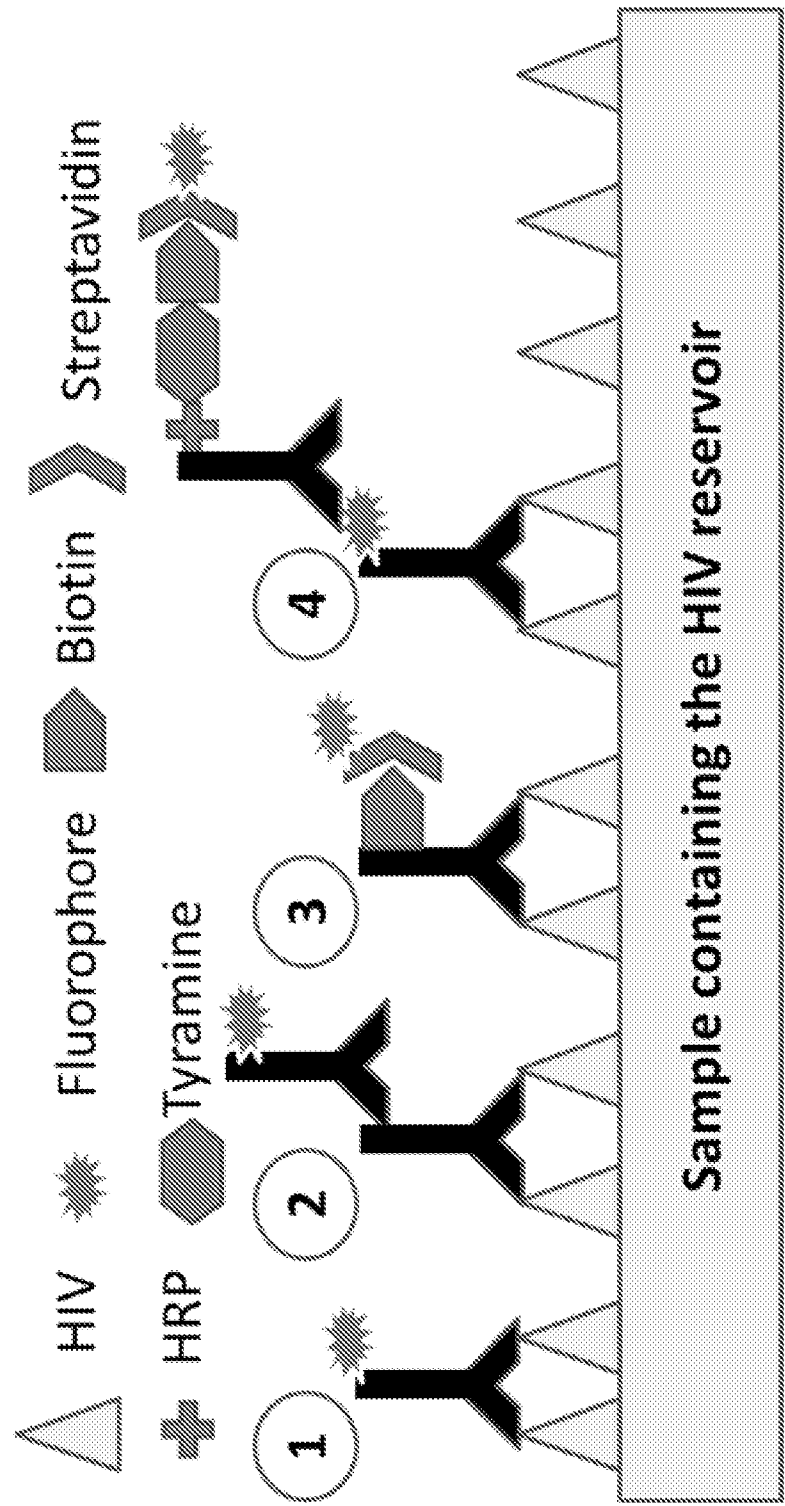


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/17535

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 9-16, 17-19, 21, 22
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/17535

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - A61B 5/00, G01N 33/53, G01J 3/28, G01J 3/457 (2015.01) CPC - A61B 5/00, G01N 33/53, G01N 33/532, G01J 3/28, G01J 3/2823 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC(8)- A61B 5/00, G01N 33/53, G01J 3/28, G01J 3/457 (2015.01); CPC- A61B 5/00, G01N 33/53, G01N 33/532, G01J 3/28, G01J 3/2823 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched USPC- 356/326, 435/4, 435/7.1, 436/171, 436/805; Patents and NPL (classification, keyword; search terms below) Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Pub West (US EP JP WO), Pat Base (AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO), Google Patent, Google Scholar, Free Patents Online; search terms: molecule, detect, identify, measure, signature, spectral, optical, light, wavelength, frequency, dye, tag, antibody, probe, laser, stain, endogenous, biotin, block, solution		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2004/0215087 A1 (GENERO et al.) 28 October 2004 (28.10.2004), para [0004], [0096], [0157], [0165], [0166], [0170], [0171], [0188], [0207], [0235], [0236]	1, 3-7, 8/(1, 3-7), 20
X	US 2003/0170613 A1 (STRAUS) 11 September 2003 (11.09.2003), para [0033], [0057], [0093], [0105], [0145], [0147], [0270], [0304], [0310], [0355]	1, 2, 8/(1, 2)
Y	US 2013/0344508 A1 (SCHWARTZ et al.) 26 December 2013 (26.12.2013), para [0013]-[1282]	1-8, 20
Y	US 2005/0244863 A1 (MIR) 03 November 2005 (03.11.2005), para [0022]-[1090]	1-8, 20
Y	US 2004/0096872 A1 (GRAY et al.) 20 May 2004 (20.05.2004), para [0073]-[0367]	1-8, 20
☐ Further documents are listed in the continuation of Box C. ☐		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 25 April 2015 (25.04.2015)		Date of mailing of the international search report 29 MAY 2015
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300		Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774