Hyperspectral imaging to monitor simultaneously multiple protein subtypes and live track their spatial dynamics: a new platform to screen drugs for CNS diseases

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ABSTRACT

In the past decade, the efficacy of existing therapies and the discovery of innovative treatments for Central Nervous System (CNS) diseases have been limited by the lack of appropriate methods to investigate complex molecular processes at the synaptic level. In order to better understand the fundamental mechanisms that regulate diseases of the CNS, a fast fluorescence hyperspectral imaging platform was designed to track simultaneously various neurotransmitter receptors trafficking in and out of synapses. With this hyperspectral imaging platform, it was possible to image simultaneously five different synaptic proteins, including subtypes of glutamate receptors (mGluR, NMDAR, AMPAR), postsynaptic density proteins, and signaling proteins. This new imaging platform allows fast simultaneous acquisitions of at least five fluorescent markers in living neurons with a high spatial resolution. This technique provides an effective method to observe several synaptic proteins at the same time, thus study how drugs for CNS impact the spatial dynamics of these proteins.

Keywords: cellular imaging, QD labelling, hyperspectral imaging, biomarkers multiplexing

1. INTRODUCTION

Despite many years of investigations, the mechanisms of central nervous system (CNS) functions are not fully understood yet. In particular, this affects diseases where multiple CNS functions are involved with complex mechanisms, such as memory, learning, cognition and neuronal plasticity. The lack of a full understanding of these mechanisms limits the development of more effective drugs for the cure of CNS diseases, such as Alzheimer, Huntington disease, Parkinson, schizophrenia, depression and autism.

In order to discover innovative treatments that would efficiently cure these diseases, it is necessary to study at the synaptic scale the complex processes that regulate memory, learning, cognition and neuronal plasticity. In fact, it is known that when diseases caused by the dysfunctions of these mechanisms occur, the spatial dynamics between proteins and their interactions are modified and induce changes in cellular functions[1].

Recent imaging studies showed that synaptic proteins are extremely dynamic and exchange between extrasynaptic and synaptic domains[2]. However, currently available imaging techniques do not allow the visualization of interactions between multiple brain targets and drugs. While one or two synaptic proteins can already followed with a fast and efficient global imaging system, a multiplex system is essential to monitor several protein receptors simultaneously. A hyperspectral imaging platform will greatly improve the understanding of the complex neuronal interactions between molecular processes and the effect of drugs on CNS diseases.

When several synaptic proteins are simultaneously labeled with multiple quantum dots (QDs), they can be identified and localized according to the emission spectra of each QD. As a consequence, these tagged proteins can be monitored simultaneously and tracked in time with similar spatial resolution and image quality as for light microscopy techniques.

In this study, we present a new microscopy multiplex hyperspectral imaging platform (MMHIP) that allows the simultaneous imaging of protein dynamics and interactions through QDs tracking.

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In order to follow multiple QDs-tagged proteins at the same time, the MMHIP was designed to acquire spectrally resolved images of large sample areas in a short acquisition time (seconds). One of the main challenges was the choice of a technology that would allow fast and efficient acquisition of data in very demanding conditions: low intensity light and rapid spatial variations. For this purpose, Bragg Tunable Filters (BTFs) were combined with a very sensitive EMCCD camera. BTFs detect simultaneously a single wavelength of the whole image and can be tuned changing the angle of incidence of the beam on the BTF. Their transmission is continuously tunable over 400 nm range with a spectral resolution of 2.5 nm. Since these filters can achieve efficiency up to 80% with unpolarized light, they are a perfect choice for non-destructive molecular analysis with high sensitivity.

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2. METHODS

2.1 Multiplex Microscopy hyperspectral imaging platform

The MMHIP is composed by three main components: microscope, hyperspectral filter and camera. The sample is illuminated with a fluorescence lamp and the signal emitted is collected on an electron multiplying charge-coupled device (EMCCD) camera after being filtered by the BTF (Figure 1).

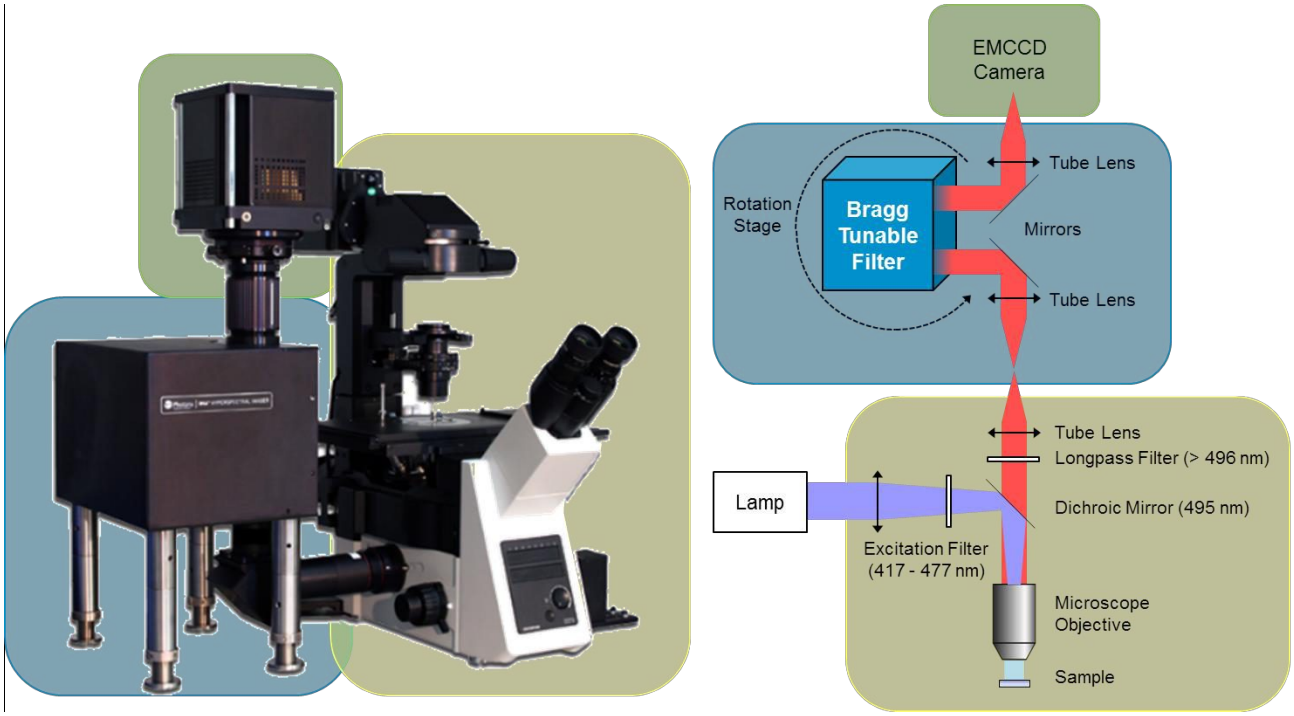

Figure 1: Scheme of the multiplex hyperspectral imaging platform with the main components: microscope (yellow background), hyperspectral filter (blue background) and camera (green background).

Microscope
QDs with three different emission spectra (Life Technologies) were dispersed on a glass slide and observed with a commercial epifluorescence microscope (IX-73, Olympus) with a 100x oil immersion microscope objective. A xenon-arc lamp (Lambda LS, Sutter Instrument) in combination with a bandpass filter (λ_{exc} = 417-477 nm) was used as illumination. A dichroic mirror (495 nm cutoff wavelength) separated the illumination from the arc lamp and the emission from the QDs. A long-pass filter (496 nm cutoff wavelength) decreased further the intensity of unwanted light from the filtered arc lamp.

Hyperspectral Filter
The hyperspectral filter was positioned at one of the available exit of the microscope, at the typical port of a camera. The full image in the field of view is sent to the BTF through two tube lenses, which are used as a relay in order to image the pupil on the BTF. When the signal emitted by the sample reaches the BTF, only a single wavelength is filtered, thus each monochromatic image exits the filter. The whole emitted signal reaches the BTF, but only the single wavelength resonant with the BTF is diffracted and transmitted, then reaches the camera, while all other wavelengths are refracted and sent away from the main optical path.

Camera
The filtered monochromatic image was then formed by a tube lens on an EMCCD camera. Wavelengths are scanned by changing the angle of incidence of the beam on the BTF. Because of its high sensitivity, a TEC cooled EMCCD camera (HNü512, Nüvü Camēras) with 512×512 pixels of 16 µm pixel size, 20 MHz was chosen.

2.2 Details about the filter

The BTF optical core is a volume hologram where the index of refraction varies periodically [3]. When a collimated polychromatic beam reaches the BTF, only a particular narrow bandwidth satisfies the Bragg condition and constructively interferes with the refractive index modulation, while all the other wavelengths are not diffracted (Figure 2, left pane). When the incident angle of the incoming light is changed, the resonant wavelength varies (Figure 2, right pane), and can be tuned over hundreds of nanometers.

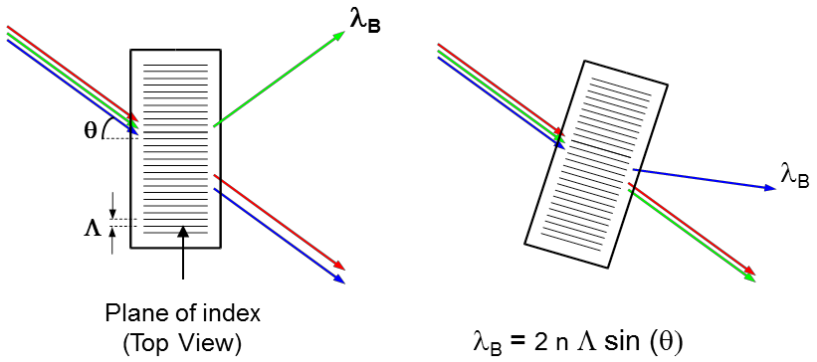

Figure 2: Filtering scheme for transmission BTF

Since the grating is also dispersive, the volume hologram generates a divergent beam for the diffracted narrow bandwidth. As showed in Figure 3, a second pass in the grating compensates for the beam divergence of the diffracted beam. An image can be reconstructed only if both optical paths within the grating are parallel. To ensure parallel optical paths, a corner cube is used to redirect the light to a second region of the grating. The second filtering step narrows down further the bandwidth, compensates for the dispersion, and delivers an out-going beam in a direction parallel to the incoming one.

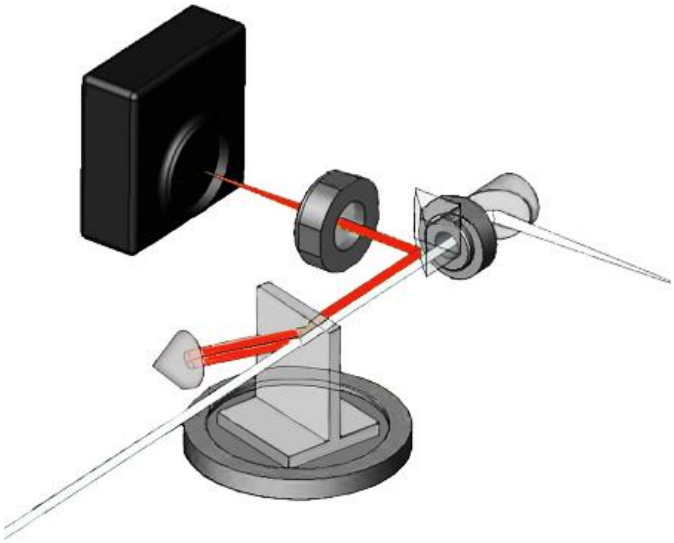

Figure 3: Current design of a transmission imaging Bragg tunable filter using a volume hologram.

To ensure that the beam entirely passes through the volume hologram, the pupil of the optical system is imaged between the two grating passes by the first tube lens. The corner cube and the volume hologram are positioned on rotation stages to tune the diffracted wavelength. The non-resonant light passes through the grating, without being diffracted. The diffracted beam is reflected and focused on an EMCCD camera by another tube lens. The optical path within the filter has been carefully designed to eliminate the variations in the outcoming beam pointing. The tunability of the filter is accurate within 50 pm. With the aid of the internal calibration system, the filter can achieve this repeatable tuning precision over the full range.

The beam at the output of the microscope can be decomposed in a sum of collimated beams issued from different positions of the object seen by the microscope objective. Each collimated beam has a different incident angle on the volume hologram. The angular selectivity of the grating leads to a gradient in wavelength across the field of view in the dimension parallel to the dispersion axis (Figure 4). To get a monochromatic image, one would need to scan through a few wavelengths and retrieve the wavelength of interest for each image. This reconstruction is routinely done using PHOTON ETC. software.

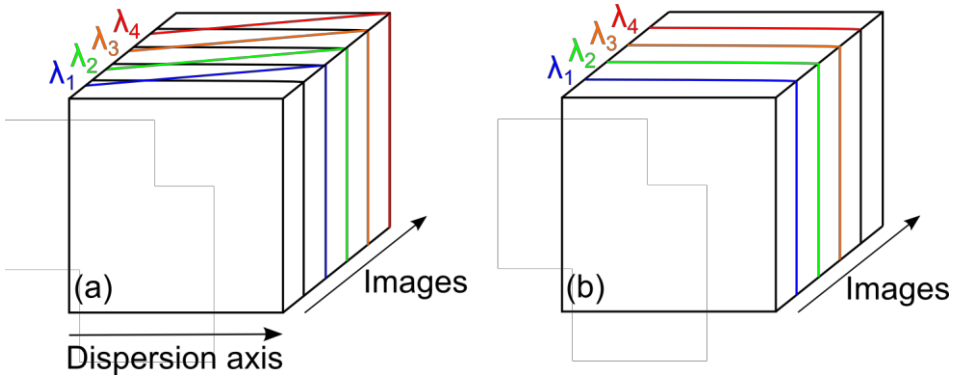

Figure 4: (a) Images with a gradient in wavelength in the dimension parallel to the dispersion axis and (b) reconstructed monochromatic images.

Volume holograms can reach diffraction peak efficiency of more than 90% (Figure 5). Figure 5a shows the calculated diffraction efficiency of a transmission volume hologram for s- and p-polarization; the grating has been designed for a maximum unpolarized diffraction at a wavelength of 671 nm. Due to optical path considerations, the incidence angle limits the diffracted wavelength between 500 to 900 nm.

Figure 5b shows the spectral response of the volume hologram used in the current set-up. It is designed to have a full width at half maximum of 2.5 nm. The double passage into the BTF further decreases the side lobes to 1%.

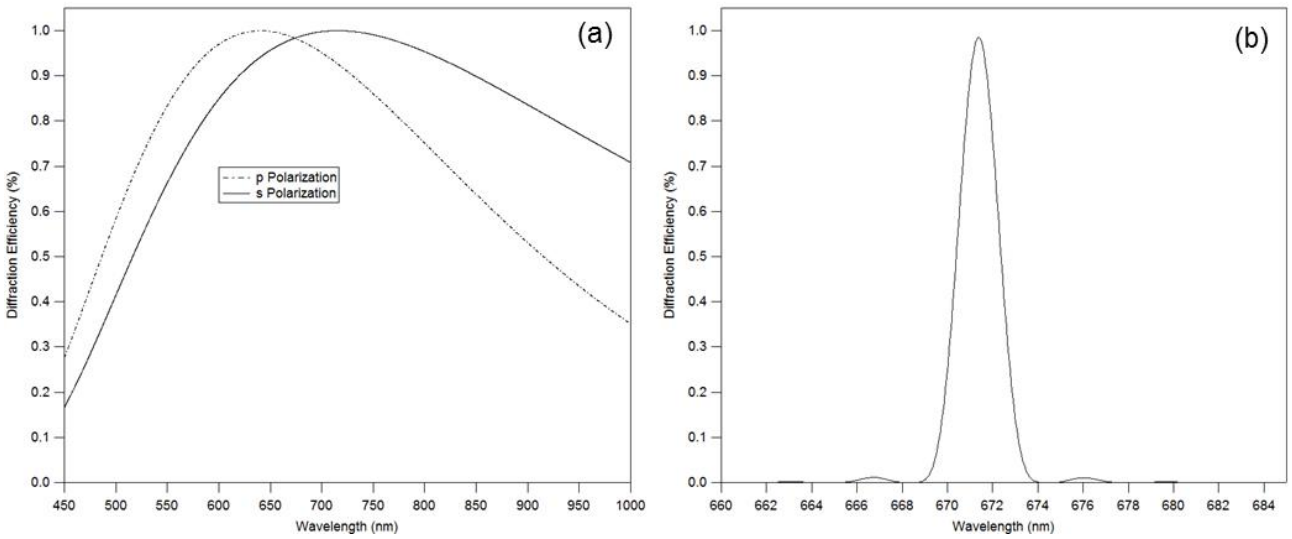

Figure 5: (a) Modeled diffraction efficiency for a reflection volume hologram for s- and p-polarization (solid and dash lines, respectively). (b) Spectral response of the volume hologram centered at 671 nm.

2. RESULTS AND DISCUSSION

The MMHIP was developed specifically for multiplex cellular imaging. As a proof of concept, three different QDs were deposed on a microscope slide and observed with a 100x objective. The intensity of the light was set at the minimum power necessary for the sample visualization. Results about the diffusion of multiple tagged synaptic proteins will be presented in a future paper.

Upon excitation centered at 447 nm, the emission of the sample was collected between 500 nm and 900 nm with a step of 1 nm and exposition time of 30 ms. As shown in Figure 6a, the different QDs can be located in the image according to their emission spectra. Since spectral information is obtained for each pixel of the image, it was possible to differentiate also adjacent QDs (inset). Each spectrum of Figure 6b is related to a different pixel indicated with the corresponding arrow in the inset. The maximum emission peak is centered at 585 nm, 605 nm, 655 nm, in agreement with the maximum emission wavelengths provided by the QDs manufacturer.

The monochromatic images of the sample at the wavelength of the emission peak are showed in Figure 7. Each image covers an area of 82 x 82 μm. In order to create a composite of the emission peak images, blue was assigned to 585 nm, green to 605 nm, and red to 655 nm. These single channels were merged and the resulting image is shown in Figure 6a.

In order to cover the gradient, the full acquisition was taken between 460 nm and 540 nm, and the hyperspectral cube of images was automatically rectified by PHOTON ETC. software to reconstruct the monochromatic images. The total acquisition time, including rectification, was less than 1 min.

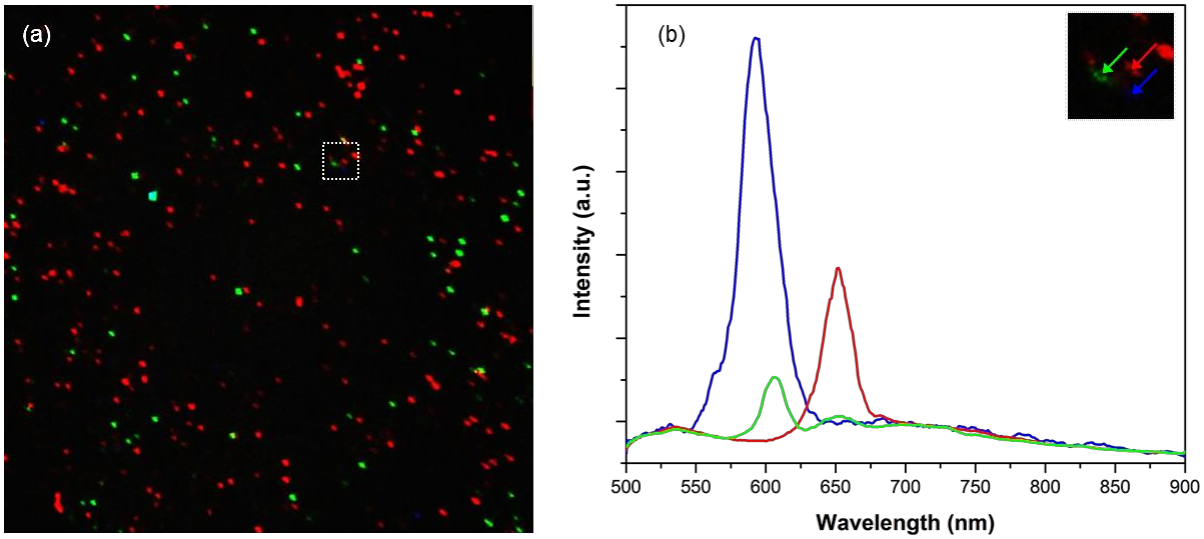

Figure 6: (a) Composite image of three QDs and their emission spectra (b).

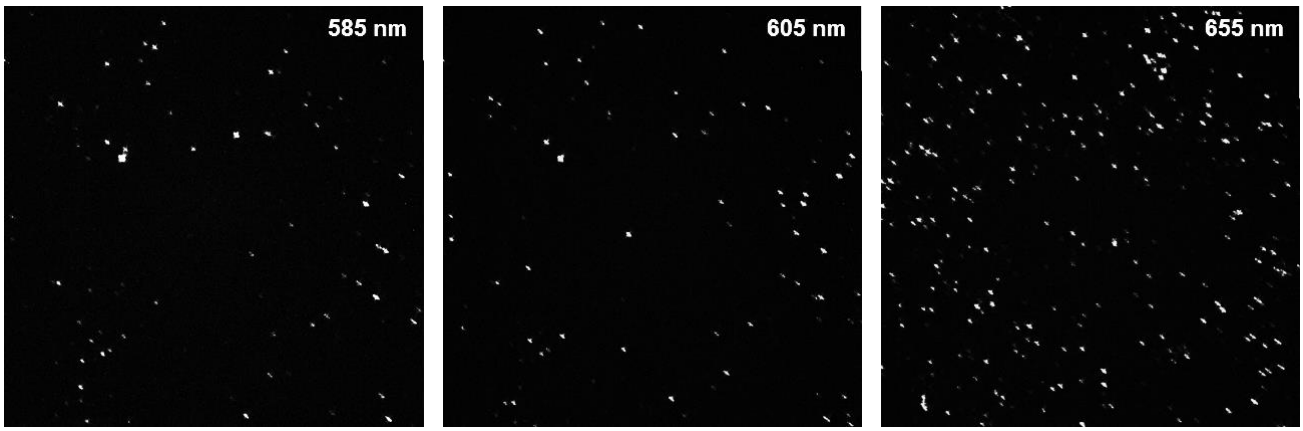

Figure 7: Monochromatic images of the three population of QDs at their respective emission peak wavelength.

Similar results (not shown here) were acquired in less than 10 s using the video mode acquisition. In this mode, the acquisition parameters, such as spectral range and step size, are the same as described earlier. Instead of stopping the BTF rotation to acquire a snapshot at each wavelength, the filter continuously moves from the initial to the final wavelength, and the camera acquires the images in video mode (67 fps). The resulting hyperspectral cube is similar to what showed in Figure 6, but the spectra are slightly noisier because of the shorter exposition time and the bandwidth is slightly larger due to the continuous movement of the filter. Since the main spectral features stay clearly discernable, this modality was paramount to study synaptic protein trajectories.

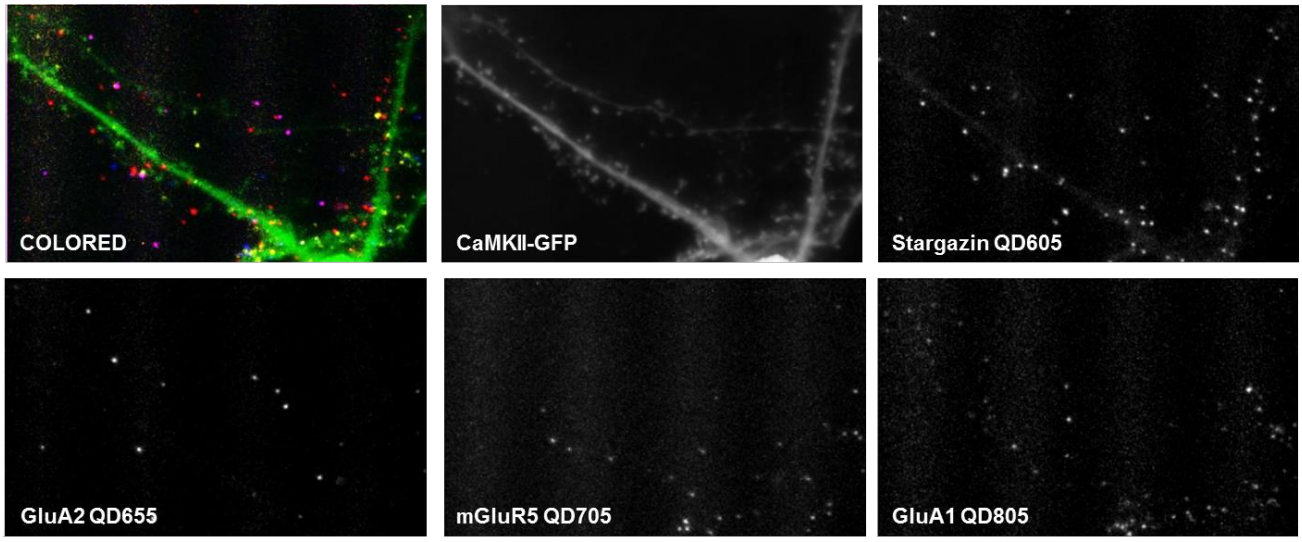

Figure 8: Detection of four membrane proteins with QDs and a synaptic marker on fixed hippocampal neurons (each image 75 X 45 μm).

Finally, in order to test the imaging system on neurons, we simultaneously overexpressed four tagged receptors, HA-Stargazin, Biotin-GluA2, Myc-mGluR5, FLAG-GluA1 and CamKII-GFP, as a synaptic marker (Figure 8). Live neurons where incubated with pre-coated specific antibodies-QD for 20 min and washed twice for 30 s before cell fixation with ethanol. We then mounted the cells on a glass coverslip with the Invitrogen Qmount mounting media (Q10336). Spectral cubes were acquired in video mode in less than 4 seconds, GFP and QDs where extracted based on their peak emissions. The images clearly show that with the MMHIP it is possible to detect neurons with single QDs with four different emission wavelengths and a GFP tagged synaptic marker.

3. CONCLUSION

A multiplex microscopy hyperspectral imaging platform was developed to study the dynamics of synaptic proteins, particularly for the mechanisms that determine CNS diseases. The MMHIP is based on Bragg Tunable Filters and is continuously tunable between 500 nm and 900 nm, with a spectral resolution of 2.5 nm. As a proof of concept, four different QDs plus a GFP tagged synaptic marker were individuated by their spectral emission on fixed neurons. The possibility of acquiring hyperspectral data at the rate of 67 fps makes the MMHIP a great tool to study rapid exchanges as those that occur in the central nervous system.

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