Multiplex protein-specific microscopy with ultraviolet surface excitation

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Abstract: Immuno-histochemical techniques, such as immunofluorescence (IF) staining, enable microscopic imaging of local protein expression within tissue samples. Molecular profiling enabled by IF is critical to understanding pathogenesis and is often involved in complex diagnoses. A recent innovation, known as microscopy with ultraviolet surface excitation (MUSE), uses deep ultraviolet (≈280 nm) illumination to excite labels at the tissue surface, providing equivalent images without fixation, embedding, and sectioning. However, MUSE has not yet been integrated into traditional IF pipelines. This limits its application in more complex diagnoses that rely on protein-specific markers. This paper aims to broaden the applicability of MUSE to multiplex immunohistochemistry using quantum dot nanoparticles. We demonstrate the advantages of quantum dot labels for protein-specific MUSE imaging on both paraffin-embedded and intact tissue, significantly expanding MUSE applicability to protein-specific applications. Furthermore, with recent innovations in three-dimensional ultraviolet fluorescence microscopy, this opens the door to three-dimensional IF imaging with quantum dots using ultraviolet excitation.

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1. Introduction

Chronic diseases such as cancer and neurodegenerative disorders are often accompanied by progressive microscopic alterations in tissue structure and protein composition. Quantitatively assessing these changes is crucial for detecting and monitoring disease [1,2]. In clinical medicine, these changes are often examined through histopathological processing, which integrates multi-step tissue preparation, sectioning, chemical labeling, and microscopy to evaluate and grade disease. However, this process is labor-intensive and time-consuming, leading to delays in diagnosis. A recent alternative, known as microscopy with ultraviolet surface excitation (MUSE), eliminates time-consuming tissue preparation and sectioning steps while maintaining the diagnostic quality of the resulting images [3]. MUSE can acquire equivalent histological images directly from fresh tissue, enabling integration into conventional histology pipelines while providing faster results at lower cost [4].

While traditional histopathological stains, such as hematoxylin and eosin (H&E), trichrome, and periodic acid-schiff (PAS) are commonly used for tissue morphology assessments, current histological practice relies on immunohistochemistry (IHC) for protein-specific labeling [5]. However, no protocols currently exist for protein-specific imaging with MUSE. To broaden the applicability of MUSE to multiplex immunofluorescence (IF), we report a framework for multiplex protein-specific MUSE labeling and imaging. We have developed protocols adopting quantum dots (QDs) as a fluorescent probe due to their unsurpassed brightness, photochemical stability, large Stokes shifts, and narrow emission bands [6–10]. In this report, we demonstrate that QD-based IHC is compatible with MUSE on both paraffin-embedded sections and intact tissue. The proposed protocols are simple to implement using commercially-available reagents.
1.1. MUSE Imaging

MUSE utilizes short-wavelength ultraviolet (UV) light (≈280 nm) to excite fluorescence in several common histological labels, allowing non-destructive imaging of the sample surface. While traditional histological procedures require multiple preparation steps, MUSE allows slide-free histology compatible with fresh tissue. MUSE also retains precious tissue samples for downstream molecular and cellular studies [3].

MUSE relies on two benefits of UV excitation. First, tissue is highly absorbant to deep UV light, significantly limiting penetration at wavelengths below 300 nm. Published studies of tissue optical properties analytically define the scattering and absorption properties that restrict light penetration for shorter wavelengths [11]. Limited penetration depth enables high contrast image acquisition from the tissue surface [12] allowing thin optical sectioning of the sample surface without the need for microtomy and slide mounting. Second, several common labels, such as 4′,6-diamidino-2-phenylindole (DAPI), Hoechst 33342 (HO342), and eosin, are fluorescent under deep-UV [13], enabling the use of several common histology labels for counter-staining. Contrary to conventional fluorescence microscopy, MUSE does not require dichroic mirrors or excitation filters since conventional glass blocks deep UV light. Only emitted photons contribute to final image formation, enabling multiplex collection with an RGB camera.

1.2. Conjugated quantum dots

QDs are semiconductor nanoparticles with optical and electronic properties that set them apart from conventional fluorescent dyes [14]. They are readily functionalized and widely used for visualizing specific biomarkers [15]. Their size-tunable light emission allows a wide range of colors with a common absorption band with large molar extinction coefficient (Fig. 1(b)). Colors are readily manipulated to collaborate with other labels for high-dimensional multiplex mapping. QDs also possess narrow emission bands with comparatively large fluorescence quantum yields [16]. Finally, QDs distinguish themselves from conventional organic fluorophores by their large fluorescence Stokes shift, which can be as large as 420 nm for red emitting QDs. Typical dyes possess Stokes shifts between 10 nm and 60 nm using conventional visible light excitation [17]. This larger Stokes shift minimizes the need for optical filtering to eliminate Rayleigh scattered light, which can significantly complicate multiplex experiments. In the case of deep UV excitation, where the emitted light is blocked by native glass optics, concurrent detection of multiple QDs is possible with an RGB color camera. Another crucial advantage of QDs in fluorescence microscopy is their relative photochemical stability under UV, unlike many conventional dyes such as DAPI. Recent work systematically investigated the photobleaching/photodestruction properties of commercially available QDs in typical biological experiments [18], demonstrating that photostability can vary significantly. As reported, the light properties of QDs depend on their structure and environment (solvent, temperature, exposure to air, etc.). Prost et al. reported a method to reduce QD photobleaching using Tris buffer with adjunction of a commercial background reducing agent (Antibody Diluent, Background Reducing, Dako Agilent).

Protein-specific labeling with QDs is often achieved through IHC processing; that is, coupling QDs with a molecule (ex. an antibody) that binds specifically to a target (ex. antigen). The chemical surface is functionalized through bio-conjugation, such as avidin/biotin and streptavidin/biotin bridging [19]. QD labeling techniques are well-established in IHC for both in vivo and in vitro experiments, which are categorized as either direct or indirect IHC [20]. In general, indirect IHC is more common in cell and tissue biology ascribable to its cost-efficiency, high-sensitivity, and signal amplification capacity. This method employs cascaded immuno-binding (Fig. 1(a)), in which an unlabelled primary antibody is used to detect the antigen of interest in the tissue and a secondary labelled antibody is used to bind exclusively to the primary antibody. The secondary antibody is attached with multiple QDs through bio-conjugation prior to binding. This study uses streptavidin/biotin recognition, in which QD streptavidin conjugates
Fig. 1. Properties of ThermoFisher QD streptavidin conjugates. (a) Using indirect IHC, the primary antibody (blue) binds the antigen and the QD-tagged secondary antibody (orange) binds to the primary. A close-up showing the structural properties of a QD streptavidin conjugate. (b) Absorption (dashed curve) and emission (solid curve) spectra of several QDs showing capability of multiplex mapping with a single deep UV excitation band. An average excitation spectrum of all QDs is plotted (black dashed curve). QDs in other sizes (ex. 525 nm, 625 nm, 705 nm) are also commercially available.

bind to a biotinylated secondary antibody. The indirect IHC method provides an intensive signal amplification over the direct IHC, which is beneficial for detecting low-abundance proteins. However, imaging performance can be hindered by cross-reactivity when performing multilabel experiments. Appropriate blocking (ex. avidin/biotin blocking) be carried out prior to antibody incubation to minimize background fluorescence.

2. Materials and methods

2.1. MUSE setup

Our MUSE imaging system (Fig. 2(a)) uses a solid-state UV source with 3 watt emission power centered at 280 nm and focused through a quartz lens with a numerical aperture (NA) of 0.25 (Phoseon Technology, Hillsboro OR). The light source is obliquely positioned 5 cm away from the sample to provide a 1 mm focal spot at the tissue surface (Fig. 2(a)) large enough for acquisition. In addition to tissue absorbance, oblique illumination minimizes excitation to superficial layers as described previously [21]. Fluorescence signals emanated from the tissue surface are collected with a 10X objective (Olympus UPLFLN10X2, 0.3NA) and relayed through a tube lens to either a 1.4 Megapixel color CCD camera (Thorlabs 1501C-GE) or a 8.9 Megapixel color CMOS camera (Thorlabs CS895CU). The effective lateral resolution is 1.29 \( \mu m \) per pixel and 0.31 \( \mu m \) per pixel respectively, verified with a standard USAF resolution target (Edmund Optics). The corresponding diffraction limit is approximately 1.02 \( \mu m \) (assuming the emission wavelength to 500 nm). The MUSE microscope is assembled with an objective turret (Thorlabs CNS500) that supports additional objectives. In this work, a 40X objective (Nikon CFI S Plan Fluor ELWD 40XC, 0.6NA) is used to achieve high-resolution down to 0.36 \( \mu m \) per pixel using the CCD camera and 0.09 \( \mu m \) per pixel using the CMOS camera. The sample holder (Thorlabs MLS203P2) is mounted to a custom three-axis motor stage (Aerotech ANT130-XY and Aerotech ANT130-L-Z) for focusing and scanning.
2.2. IHC labeling with quantum dots

QD nanocrystal streptavidin conjugates with cadmium selenide (CdSe) or cadmium telluride (CdTe) cores were purchased from ThermoFisher Scientific (QDs nanocrystals). C57BL/6 wild-type mice were rendered unconscious using CO\textsubscript{2} inhalation based on guidelines provided by the American Veterinary Medical Association (AVMA). Mice were perfused transcardially with 20mL of room temperature phosphate-buffered saline (PBS) followed by 20mL of an alcohol-based fixative, Accustain (Sigma-Aldrich). Perfusion with a formalin-free fixative is necessary to avoid antigen retrieval. Brains were dissected and immersed in Accustain. Fixed brain samples were paraffin embedded and coronally sectioned at 20\textmu m (or manually at 1 mm for free thick sections) and mounted onto charged microscope slides. This is the starting point for most biology laboratories where paraffin-embedded tissue is profusely available. The proposed QD-based immunolabeling protocol is compatible both with paraffin-embedded tissue and intact tissue.

Paraffin sections were deparaffinized using ethanol and xylene substitute (Sigma-Aldrich A5597). Deparaffinized and free sections were treated with a permeabilization/blocking buffer containing 5% normal goat serum and 0.05% Tween 20 in 1X Tris-buffered saline (TBS, 10 mM Tris-HCl, 150 mM NaCl) for 1 hour at room temperature. Brain sections were then co-incubated in primary antibodies for NeuN (1:250, mouse monoclonal; Millipore) and Collagen IV (CollIV) (1:250, rabbit polyclonal; Rockland) in blocking buffer overnight at 4 °C. The next day, sections were washed three times with 0.05% Tween 20 in TBS (TBST). Sections were then incubated first with a biotinylated goat anti-mouse secondary antibody (1:200, Jackson ImmunoResearch) in TBST for 30 minutes at room temperature. Sections were then washed once in TBST and then twice in a QD compatible buffer (1:50 Agilent Dako antibody diluent in 50 mM Tris pH 8) as described above. QD streptavidin conjugates were centrifuged for 3 minutes at 3000g just before each use then diluted 1:25 in QD buffer. Sections were then incubated in QD 585 streptavidin conjugate solution for 1 hour at room temperature. Sections were then washed three times in QD buffer (QD buffer was used for all subsequent washes). Before treating with the next secondary, the sections were blocked for avidin/biotin (Vector) as per the manufacturer’s instructions. Sections were incubated with a biotinylated goat anti-rabbit secondary antibody (1:200, Jackson ImmunoResearch) in pure Dako antibody diluent for 30 minutes at room temperature followed by three washes in QD buffer and incubation with QD 605 streptavidin.
conjugate solution as described above. Sections were then given another avidin/biotin blocking before being incubated with the third primary antibody for GFAP (1:500, rabbit polyclonal; Biolegend) in pure Dako antibody diluent overnight at 4 °C. The next day, sections were washed three times and then incubated with biotinylated goat anti-rabbit secondary antibody (1:200) in pure Dako antibody diluent for 30 minutes at room temperature. Sections were then incubated with QD 655 streptavidin conjugate solution for 1 hour at room temperature followed by three washes. Additional QDs can be integrated sequentially following similar processes as needed, or in parallel with appropriate selection of primary-secondary pairs. Depending on the application, some sections were counter-stained with other UV-excitable dyes such as HO342 according to the manufacture’s instructions.

2.3. Fluorescent ink perfusion

Briefly as described above, mice were deeply anesthetized using CO₂ and perfused transcardially with 20 mL of room temperature PBS solution followed by 20 mL of Accustain. To obtain a continuous stain of the entire vascular system, mice were then perfused a third time with 10 mL of vascular stains [22]. Multiple vascular stains were used, including India ink (Higgins) and fluorescent tattoo ink (Skin Candy) depending on the application.

3. Results

3.1. Ultraviolet excitation of quantum dots

UV excitation is compatible with QDs, where different structural properties induce fluorescence at different wavelengths with a single 280 nm excitation. GFAP was labelled with four different QDs in paraffin-embedded brain sections and imaged unmounted but moist with wash buffer (Fig. 3). The proposed MUSE microscope was compared to wide-field fluorescence (Nikon Eclipse TI-E Inverted Microscope) to demonstrate the advantages of UV excitation. Images were collected through a Nikon 40X objective providing a lateral resolution of 0.36 µm per pixel for the MUSE setup and 0.18 µm per pixel for the inverted microscope. This sampling resolution is sufficient to reveal the cell body of each individual astrocyte.

![Fig. 3. Examining QDs with deep-UV excitation. GFAP-positive cells were labelled with different QDs (left to right) that emit respectively at 565 nm, 585 nm, 605 nm, and 655 nm and imaged with a wide-field fluorescence microscope (top) and MUSE (bottom).](image-url)
While MUSE allows simultaneous acquisition of multiple fluorescent channels at the tissue surface, conventional fluorescence microscopy requires label-specific matched filters (with dichroics) for identification. For example, a custom filter cube including a 390 nm excitation filter, a BFP dichroic mirror, and a 565 nm emission filter was used to observe QDs that emit at 565 nm. This unfortunately excludes tissue anatomic information such as vascular demarcation that are visible with MUSE (Fig. 4).

![Image of tissue section labeled with QDs for different proteins](image)

**Fig. 4.** Protein-specific triplex MUSE imaging of a coronal paraffin-embedded mouse brain section, in which NeuN-positive neurons (yellow) were labeled with QD 585, CollIV-positive vessels (orange-brown) were labeled with QD 605, and GFAP-positive astrocytes (bright red-pink) were labeled with QD 655. Different tissue regions such as the hippocampal formation (denoted as HPF) and thalamus (denoted as TH) are visible under UV excitation, and individual cell groups such as medial habenula (denoted as MH) and lateral habenula (denoted as LH) are also distinguishable in the epithalamus areas. Anatomical details such as corpus callosum (denoted as CC) and myelinated fiber tracts are resolved and rendered in blue via tissue autofluorescence under UV light.

The use of deep UV excitation (1) offers sufficient excitation for all QDs, (2) restricts the excitation volume to the tissue surface, and (3) reveals additional tissue details that do not have
contrast under visible light. Images collected with MUSE possesses dramatically improved contrast and sharpness since UV penetration is significantly limited by wavelength-dependent tissue interactions [23], providing additional axial resolution through enhanced optical sectioning. This is reflected by the body shape and size of the GFAP positive astrocytes (Fig. 3).

### 3.2. Multiplex protein-specific imaging on paraffin-embedded tissue

We demonstrate protein-specific MUSE by imaging two triplex QD-labeled data sets (Fig. 4 and 5). In Fig. 4, the imaged sample is a thin (20 µm) alcohol-fixed paraffin-embedded section of the mouse hippocampus stained successively with three QDs. NeuN-positive neurons, collagen IV (CollIV)-positive vessels, and GFAP-positive astrocytes were labeled respectively with QD 585, QD 605, and QD 655 streptavidin conjugates (Section 2.2). The tissue section was imaged at a lateral resolution of 1.29 µm per pixel using 200 ms exposure with a digital gain of 100 using the CCD camera, and image corrections were performed using ImageJ. Spatial sampling resolution is sufficient to resolve neuronal nuclei, microvessels, and astrocytes. Anatomical details, such as myelin tracts, were detected under UV illumination and appeared as light white/blue features in the background. As for Fig. 5, GFAP-positive astrocytes were labeled with QD 585, myelin basic protein (MBP)-positive myelin was labeled with QD 605, and NeuN-positive neurons were labeled with QD 655 in the hippocampus. The thin tissue section (10 µm) was imaged at a lateral

![Protein-specific triplex MUSE imaging of a coronal paraffin-embedded mouse brain section, in which GFAP-positive astrocytes were labeled with QD 585, MBP-positive myelin was labeled with QD 605, and NeuN-positive neurons were stained with QD 655 in the hippocampus. Colors were manually edited to enhance image contrast.](image)
resolution of 0.31 µm per pixel using 100 ms exposure with a digital gain of 20 using the CMOS camera. This image was manually edited to enhance chromatic contrast using ImageJ.

3.3. Multiplex imaging on intact free-floating tissue

We provide an example of combining QDs with conventional histological stains using a thick (1 mm) alcohol-fixed mouse brain section sliced with a stainless-steel mouse brain slicer (Zivic instruments). The mouse was perfused with India ink, and a thick brain section was immunolabeled with QD 585 for GFAP, and counter-stained with HO342 for nuclear contrast for multiplex MUSE imaging. This image was collected at a lateral resolution of 0.36 µm using 200 ms exposure with a digital gain of 100, and was manually corrected using ImageJ to enhance brightness and contrast. Sub-cellular details, such as chromatin distribution, are visible (Fig. 6). This demonstrates the compatibility of QDs with conventional staining and 280 nm UV light excitation for MUSE imaging, and emphasizes the potential of QDs in multiplex tissue imaging with excitation restricted to the tissue surface.

![Coronal MUSE imaging of a thick intact brain section stained with three different types of stains. GFAP-positive astrocytes (denoted as A) were labeled with QD 585, all cell nuclei (denoted as C) were counter-stained with HO342, and vessels (denoted as V) were stained with India ink during lumen perfusion.](image)

4. Discussion and conclusion

MUSE is an emergent technology that enables rapid slide-free histology on fresh tissue to generate high contrast, subcellular resolution images. While studies of MUSE basics, such as underlying physical phenomena and instrument specifications, have been established [3,21], the reported applications of MUSE have been limited to conventional histological dyes [4,24]. Furthermore,
complex immuno-profiling and automated multiplexing are challenges being addressed to prepare for next-generation precision medicine and digital analysis. To fulfill this aim, we report protocols that utilize QD-based IF to realize protein-specific MUSE imaging. We demonstrate that QD-based immunolabeling fully outlines specific structures, such as neuronal nuclei and astrocytes. Furthermore, QD-based multiplex labeling reveals additional molecular and cellular details that are potentially translatable for clinical applications [25]. Current implementations of MUSE allow multiplex QD mapping using a single excitation source, optimizing imaging performance and throughput. The proposed framework allows rapid imaging of QD-labeled proteins at the tissue surface at sub-micrometer resolution using an RGB color camera, which can be readily adapted to conventional histology pipelines or clinical setups. Since many common dyes are not UV-excitable, additional excitation wavelengths can be added to the light path to meet these requirements.

Finally, we demonstrate multiplex protein-specific triplex MUSE imaging as well as compatibility with other common MUSE histology labels. These experiments show the viability and robustness of QD-based MUSE for protein-specific imaging. Multiplex QD mapping provides not only cellular details consistent with standard IHC but also rare molecular information [26] that can help identify and grade disease. This work broadens the applicability of MUSE and serves as a stepping stone for future studies that integrate QDs for bioanalytical applications. The current implementation of MUSE is fundamentally limited to two-dimensional images. However its methodology holds great potential for three-dimensional implementation and we have investigated this possibility and constructed an imaging platform named milling with ultraviolet excitation [27]. Using tissue clearing methods such as CLARITY [28] and whole-organ labelling strategies [29], we expect to be able to perform IF-MUSE imaging in whole-organs. To this end, tissue will be labeled with multiple QDs via stochastic electrotransport and embedded in paraffin wax for three-dimensional imaging and sectioning using our new system.

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**Disclosures**

The authors declare that there are no conflicts of interest related to this article.

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