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Real-time imaging of exocytotic mucin release and swelling in Calu-3 cells using acridine orange

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Abstract

Mucus secretion is the first-line of defence against the barrage of irritants inhaled into human lungs, but abnormally thick and viscous mucus results in many respiratory diseases. Understanding the processes underlying mucus pathology is hampered, in part, by lack of appropriate experimental tools for labeling and studying mucin granule secretion from live cells with high sensitivity and temporal resolution. In this report we present original spectroscopic properties of acridine orange (AO) which could be utilized to study granule release and mucin swelling with various advanced fluorescence imaging approaches. Low concentration ($<200 \mu\text{M}$) AO solutions presented absorption maximum at 494 nm, emission maximum at 525 nm and only ~ 1.76 ns fluorescence lifetime. By contrast at high concentrations (4–30 mM) favoring formation of AO aggregates, a very different absorption with maximum at ~ 440 nm, dramatically red-shifted emission with maximum at 630 nm, and over 10-fold increased fluorescence lifetime (~ 20 ns) was observed. To verify potential utility of AO for real-time imaging we have performed confocal, total internal reflection fluorescence (TIRF) and fluorescence lifetime imaging (FLIM) of AO-stained Calu-3 cells. We found similar red-shifted fluorescence spectra and long fluorescence lifetime in intracellular granules as compared to that in the cytoplasm consistent with granular AO accumulation. Mechanical stimulation of Calu-3 cells resulted in multiple exocytotic secretory events of AO-stained granules followed by post-exocytotic swelling of their fluorescently-labeled content that was seen in single-line TIRF images as rapidly-expanding bright-fluorescence patches. The rate of their size expansion followed first-order kinetics with diffusivity of $3.98 \pm 0.07 \times 10^{-7} \text{ cm}^2/\text{s}$, as expected for mucus gel swelling. This was followed by fluorescence decrease due to diffusional loss of AO that was ~ 10 -fold slower in the secreted mucus compared to bulk aqueous solution. In summary, we showed that AO-staining could be utilized for real-time

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TIRF imaging of mucin granule exocytosis and mucin swelling with high sensitivity and temporal resolution. Considering unique AO fluorescence properties that permit selective excitation of AO monomers versus aggregates, our study lays the groundwork for future development of two-color excitation scheme and two-color fluorescence FLIM live-cell imaging assay with potentially many biological applications.

Keywords

Acridine orange; Spectral shift; Fluorescence lifetime; Total internal reflection fluorescence; Mucin granules; Cystic fibrosis

1. Introduction

Mucus provides a physical barrier that protects the moist surfaces of the body from harsh environment, toxins and pathogens. In the lung mucus forms a thin film of viscoelastic gel on the surface of the airways that entraps foreign debris, bacteria, and viruses inhaled during breathing and helps removing them by mucociliary clearance (MCC) process. The MCC requires that mucus retains specific rheological properties allowing for its transportability, i.e., ability to be moved by the tips of beating cilia. In disease conditions, such as cystic fibrosis (CF), asthma, and chronic obstructive pulmonary disease, excessive production of mucus and changes of its viscoelastic properties, result in accumulation of thick, sticky mucus in the lungs, effectively impairing the MCC process. This leads to difficulty in breathing, chronic infections, morbidity, and in severe cases, mortality [1]. The major macromolecular component of the mucus are polymeric mucins (MUC2, MUC5AC, MUC5B, MUC6, and MUC19) [2,3], a family of large, disulfide-bonded glycoproteins, which due to heavy glycosylation can exhibit a molecular mass in the range of MDa. These macromolecules are stored in cytoplasmic granules in a highly condensed state in which high Ca^{2+} concentration and acidic pH counteract the repulsive forces of mucin's fixed polyanionic charges [4]. Upon stimulation mucin granules undergo rapid exocytosis, in tens of millisecond, followed by release of their cargo. This is accompanied by rapid hydration and enormous several-hundred-fold expansion of secreted mucins, typically in several hundred milliseconds [1]. Mucin swelling is governed by a Donnan equilibrium process, and its radial expansion follows first-order kinetics [5]: $r(t) = r_1 e^{-t/\tau}$ – where r_1 is final radius after expansion, τ – time constant. Despite its importance in airway physiology and pathophysiology, our understanding of a complex multistep mucus release process and its regulation still remains rudimentary. In particular, relatively little is known about the dynamics of intra-granular $[\text{Ca}^{2+}]$ and pH changes following mucin granule recruitment and fusion with the plasma membrane, as well as sequential events underlying the release and solvation of densely-packed mucin macromolecules that is required for the formation of a thin sheet of gel on mucosal surface. Viscoelastic properties and conformation of secreted mucins strongly depend on their dielectric properties, counterion composition and pH [6,7]. In addition, small anions particularly HCO_3^- may have critical role in these processes especially in CF, where defective function of CFTR anion channel results in abnormal HCO_3^- transport and may contribute to mucus pathology [8–11]. However, direct effects of

CFTR function and different ions in the cytoplasmic and extracellular environment on mucin secretion and its viscoelastic properties have not been investigated in much detail.

Mucin secretion process could be most directly studied by real-time imaging of single mucin granule exocytosis and examining kinetics of post-exocytotic mucin swelling in live cells. However, progress in this area of investigations is hindered by lack of suitable mucin stains and imaging methods. The technical challenges arise from the fact that secretion and post-exocytotic swelling processes are very rapid, often fully completed in few-hundreds of milliseconds, requiring high rate of image acquisition, high sensitivity and spatial resolution. Phase contrast video microscopy was used in the previous studies, but at microscopic level mucus is transparent resulting in limited sensitivity and spatial resolution [1,6]. Fluorescence microscopy offers the advantage of superior sensitivity and improved spatial resolution, but shortage of appropriate fluorescent dyes, especially dyes sensitive to mucin volume and density change limits the progress.

To gain mechanistic insights into the mucin secretion process, we have evaluated several dyes for their ability to stain mucin granules in Calu-3 cells and found that acridine orange (AO) exhibits very interesting and potentially very useful properties that can be utilized for studying secretion processes. AO is a widely used metachromatic stain for nucleic acids, when it intercalates with DNA it fluoresces green (525 nm), and when it interacts with RNA it fluoresces red (>630 nm) [12]. Such spectral shift is also observed when AO accumulates at high concentrations in acid intracellular compartments and is attributed to the formation of di- and oligomeric aggregates [13–15]. Although AO is one of the oldest fluorescent dyes, its properties at high concentrations favoring aggregated form are still largely unknown, including extinction coefficient or quantum yield. Lack of such information might be attributed to the fact that these parameters are very difficult to properly measure at high dye concentration.

In this report we describe a novel approach to measure absorption and emission spectra, quantum yields and fluorescence lifetimes of AO at saturating concentrations and evaluate these parameters in the context of cellular imaging. We have also verified utility of AO for real-time imaging of mucin granules in Calu-3 cells with total internal reflection fluorescence (TIRF) microscopy. Although TIRF microscopy was used previously to study FM 1–43 labelled granules mobility and exocytosis in pancreatic duct epithelial cells, to our knowledge real-time studies of postexocytotic swelling kinetics of secreted mucins by this technique was never attempted [16]. Here, using such an approach we were able to follow the kinetics of single mucin granule release and swelling of its cargo with unprecedented sensitivity and high spatial and temporal resolution. This method also allows evaluation of AO diffusivity in secreted mucin patch, providing complementary information on mucus rheology. Unique AO fluorescence properties described here, permit selective excitation and visualization of AO monomers and aggregates and thus open new opportunities for future development of a highly-sensitive real-time mucin secretion assay based on two-color interleave excitation scheme and two-color fluorescence lifetime imaging (FLIM) detection.

2. Materials and methods

2.1. Solutions and chemicals

Physiological isotonic solution (PS) contained (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 glucose and 10 TES, pH 7.4, adjusted with NaOH. In spectrophotometry experiments phosphate buffer solution (PBS) was used and contained (in mM): 136.89 NaCl, 2.68 KCl, 10.13 NaHPO₄, 1.76 KHPO₄. Mucin from porcine stomach Type III (cat. # M1778), and acridine orange (AO) was obtained from Sigma Aldrich (St. Louis, MO).

2.2. Cells

Human lung adenocarcinoma Calu-3 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and were grown in Eagle's minimal essential medium with Earle's balanced salt solution (EMEM, ATCC) with 2 mM L-glutamine and 15% fetal bovine serum (FBS). For imaging experiments cells were grown on glass coverslips, or on collagen-coated, permeable supports (1 µm pores PET transparent membranes) hanging cell culture inserts ø24 mm, Millipore cat. # PIRP30R48, and were cultured at the air-liquid interface for at least 10 days post-confluence. For fluorescence labeling cells were incubated at room temperature for 10–20 min in culture media containing 5–10 µM AO. Dye loading was not different at 37 °C. After wash, for confocal and FLIM microscopy cells were placed in PS solution in the imaging chamber maintained at room temperature. For TIRF imaging of mucin secretion cells were placed in heated (37 °C) imaging chamber filled with 1 ml of PS. To induce mucin secretion cells were stimulated mechanically by brief gentle pressing of the coverslips, or filter membrane, with cells face-down against the imaging chamber glass bottom. In some experiments with cells grown on porous filters, mucin secretion was stimulated by thrombin (7.5 U/ml), a protease-activated agonist.

2.3. Immunostaining of secreted mucins

After stimulated secretion mucins that remained on the glass substrate of the TIRF imaging chamber were fixed *in situ* with Tissuefix solution containing neutral buffered formaldehyde (Laboratory Gilles Chaput Inc., QC, Canada) for 10 min at room temperature. After wash with PBS containing 0.5% FBS a primary monoclonal antibody against MUC5AC (clone 45M1, shows immunoreactivity with human and mouse MUC5AC), Invitrogen Corp., (Grand Island, NY, USA; cat. #18–2261) was added and incubated for 1 h at room temperature. Following 3 × wash with PBS (0.5% FBS) secondary antibody Alexa Fluor 488-labelled donkey antimouse IgG was applied for 30–60 min. Fluorescence images (at 519 nm) were acquired with Zeiss AxioObserver Z1 inverted microscope using LED illumination Colibri system (excitation 495 nm).

2.4. Fluorescence spectra and lifetime measurements

For absorption and steady state fluorescence measurements, an Agilent 8354 spectrophotometer, Cary Eclipse spectrofluorometer (Varian Inc., Australia), and FluoTime 300 (PicoQuant GmbH, Germany) were used. For time-resolved (lifetime) measurements, FluoTime 200 and FluoTime 300 (PicoQuant GmbH, Germany) time domain (time-correlated single photon counting, TCSPC) spectrofluorometers were used. Both time-

resolved spectrofluorometers contain multi-channel plate detectors (PMTMCP, Hamamatsu, Japan) and laser diodes LDH-PC-470 and LDH-PC-480 with pulse width of <70 ps as the excitation sources. In addition the FluoTime 300 can be supported with a super-continuum light source Fianium SC400-4 equipped with Aviv monochromator that allows continuous wavelength selection in the range between 400 nm and 900 nm with pulses width of ~6 ps. The fluorescence decays were fitted with FluoFit version v-4.0 software (PicoQuant GmbH, Germany) using a multiexponential deconvolution model. We studied AO properties at various concentrations and pH. To mimic intra-granular acidic environment AO was dissolved in PBS pH 5. In some experiments PBS containing 0.05 mg/ml mucin type III from porcine stomach was used to examine the effect of mucins on AO fluorescence.

2.5. Fluorescence microscopy and analysis

Loading of AO into Calu-3 cells and its intracellular distribution were studied by time-resolved confocal fluorescence imaging microscopy (FLIM). Fluorescence lifetime, the average time that the fluorophore molecule spends in the excited state, is strongly affected by the AO concentration. Therefore, FLIM could be an exceptionally sensitive method to map different intracellular fluorophore accumulation that will provide information complementary to fluorescence intensity images. The time-resolved images were obtained on Micro-Time 200 (Picoquant, GmbH, Berlin, Germany) confocal microscopy system. Excitation was provided by a 470-nm pulsed diode laser (PicoQuant) operating at a 20 MHz repetition rate; it was reflected off a 490 nm dichroic plate into an Olympus IX71 inverted microscope and passed through an Olympus 60 × 1.2 NA objective. The laser spot remained stationary while the sample was precisely positioned with a piezoelectric stage (Physik Instrumente, Karlsruhe, Germany). The emitted light after passing through the major dichroic plate was filtered through a 488-nm long wave pass, interference filter and then through a 30-μm confocal pinhole. An additional 510/23BP filter (Shamrock Inc., Rochester, NY) was used for lifetime measurements. The light path terminated at a hybrid photomultiplier (Hamamatsu) detector assembled by PicoQuant. The time response of this detector, including the width of the laser pulse, was estimated to be <120 ps. The signal from the detector was routed into a PicoHarp 300 (PicoQuant) time-correlated single-photon counting module which time-tagged each photon. All analysis was performed by the SymPhoTime, v. 5.3.2 software from PicoQuant.

Exocytosis of AO-stained mucin granules was studied by time-lapse TIRF imaging with an Olympus IX71 inverted microscope equipped with oil immersion objectives 60x TIRF (NA 1.45), or 100x TIRF (NA1.49), under through-the-objective evanescent field illumination, as described in [17]. The microscope was set into TIRF mode by looking at 100× diluted suspension of 0.1 μm fluorescent microspheres (Molecular Probes F8800, FluoSpheres® Carboxylate-Modified Microspheres, 0.1 μm, Orange Fluorescent, 2% solids). The TIRF mirror was adjusted until microspheres begun to flicker, i.e. they would briefly appear and rapidly disappear from the field-of-view. This indicated that TIRF angle was correctly adjusted, i.e. spheres briefly entered TIRF volume and rapidly diffused away from it. If the angle was too large, nothing could be seen. If the angle was too small, there was no TIRF volume and many spheres could be seen with low contrast and no flicker.

During experiments with cells fluorescence was excited with the 488-nm line of an argon air-cooled laser IMA101010-B0S (Melles Griot): output power was 65 mW. Laser light intensity was reduced by 50–75% with a neutral density filter. Images were acquired via a 512 nm emission filter typically at the rate of 8 to 13 image/s and exposure time of 30 ms with a Hamamatsu EMCCD camera (C4742–95) controlled by HC Image acquisition software (Hamamatsu Corp., Sewickley, PA, USA). Part of the experiments were performed with Zeiss AxioObserver Z1 inverted microscope equipped with oil immersion Alpha Plan-Apo 63x/1.46 objective, motorized Laser TIRF 3 system (Zeiss), 488 nm laser, and Evolve 512 EMCCD camera (Photometrics, USA). For TIRF imaging glass coverslips, or filter membrane, with fluorescently-labeled cells were washed with PS and placed up-side-down with cells facing the glass bottom of the imaging chamber, Fig. 1. The imaging chamber was mounted on a heated (37 °C) platform (PH-1, Warner Instruments, Hamden, CT) on the stage of the Olympus microscope, or microincubator (PM S1, Pecon GmbH, Erbach, Germany) for Zeiss microscope. When cells were grown on permeable supports, the ø24 mm filter membrane was cut-out and divided into 4–6 small pieces that were used in separate experiments. Small glass rod was placed on top of the coverslip or membrane with cells, to prevent floating and to assure close contact between cell's apical membrane and surface of the imaging chamber glass bottom. This was essential in TIRF imaging to assure that the cell's tops were within the evanescent field, extending typically ~100 nm from the upper surface of the glass chamber bottom, Fig. 1.

2.6. Analysis of post-exocytotic mucin swelling

TIRF images were analyzed off-line with MetaMorph 7.5. Circular regions of interest (ROIs) were manually placed on area showing secretory events and average intensity for each ROI was measured in the sequential stack of images. To analyze secretory event as a fluorescence area changes, the patch area was determined from number of pixels those fluorescence intensity exceeded given threshold, which was set at the baseline intensity before secretion, typically 20,000 AU under our experimental conditions. The size of each individual ROI was adjusted so, that at the peak, it will encircle all pixels that showed intensity above the baseline/threshold level. Radial mucin patch expansion was analyzed assuming a first-order kinetics: $r(t) = r_1 e^{-t/\tau}$, where r_1 is final radius of the mucin patch after expansion, $r(t)$ – radius at time t , τ – time constant, t – elapsed time. The fit was performed with Origin 7.5. To analyze diffusion of AO after its exocytotic release, intensity profile along a line across the release site was measured using a linescan feature in the MetaMorph software (see Fig. 11), at different time points after secretion. The intensity profile was corrected for the baseline, typically 3–5 linescans before the secretory event, and the intensity distribution was fitted with a Gaussian distribution (Origin 7.5):

$$y(t) = y_0 + \frac{A}{w \sqrt{\frac{\pi}{2}}} e^{-2(x-x_c/w)^2}, \text{ where } w = 8Dt.$$

3. Results and discussion

3.1. Absorption

Measurements of AO absorption at low concentrations were done using standard cuvettes of various thicknesses (5 cm, 1 cm and 0.5 mm). For concentrations above ~0.6 mM due to

high absorption sandwich type samples were used with solutions of AO squeezed between two # 1 microscopy slides Menzel-Gläser (Gerhard Menzel GmbH, Braunschweig, Germany) separated by calibrated stainless steel (or Teflon) shims. For the highest concentrations close to the solubility limit (~ 105 mM), the samples were squeezed without the shim to limit the layer thickness to ~ 1 – 2 microns. We have to stress that with such samples and those with calibrated shims of <10 microns, slide deformation by surface tension often resulted in non-uniform layers, making control of actual thickness difficult. The normalized absorption spectra for various concentrations (10 μ M to 105 mM) are presented in Fig. 2A. For low concentrations favoring monomeric form of AO a long wavelength absorption band with maximum at 494 nm dominates, as reported earlier [18–20]. For concentrations above ~ 0.1 mM a short-wavelength shoulder emerges and for concentrations above ~ 0.3 mM a short wavelength absorption band starts taking over indicating appearance of AO aggregates. For concentrations higher than 1 mM the spectrum is dominated by short-wavelength band with maximum progressively shifting to 450 nm. Thus, relative fraction of excited monomers and dimers will depend on the excitation wavelength. To illustrate this we calculated ratio of normalized absorption spectra of dimer to monomer respectively (Fig. 2 B), which reveals maximum at ~ 435 nm and minimum at ~ 494 nm. It also shows that excitation at 494 nm will preferentially excite monomeric form while 435 nm excitation will preferentially excite aggregated form under conditions where both forms are present.

3.2. Emission

The emission spectra for lower AO concentrations corresponding to monomeric form of the dye can be conveniently measured in standard square geometry using cuvettes of 1 cm to 0.5 mm thickness. For concentrations above 3 mM due to high absorption front-face configuration [21] must be used. Normalized emission spectra of AO at concentrations ranging from 10 μ M to 100 mM in water excited at 494 nm and 435 nm are shown in Fig. 3 A and B respectively. The lowest concentration shows emission spectrum with maximum at about 525 nm while highest concentration shows emission spectrum with maximum at 640 nm. The quantum yield measured against fluorescein (pH 9) was calculated to be 0.155 ± 0.003 for monomeric AO at concentrations below 1 mM. Quantum yield of aggregated form (at 100 mM) was measured using highly concentrated $\text{Ru}(\text{bpy})_2$ water solution as a reference standard and it was found to be 0.015 ± 0.002 assuming $\text{Ru}(\text{bpy})_2$ quantum yield of 0.02 .

For the lowest and highest AO concentrations used here the emission spectra were independent of excitation wavelength demonstrating that predominantly a single form, i.e. monomeric or aggregated respectively, were present in the solution. The intermediate concentrations contain mixture of monomeric and aggregated forms in various proportions and their emission spectra were strongly dependent on the excitation wavelength. As predicted from absorption spectra shown in Fig. 2, excitation at 435 nm preferentially excites aggregated form while 494 nm preferentially excites monomeric form. To illustrate this, examples of emission spectra measured for the two excitation wavelengths with 2 mM and 7.5 mM AO are shown in Fig. 4 A and B respectively. For 2 mM concentration excitation at 494 nm reveals almost pure monomeric form while for 7.5 mM concentration and excitation of 435 nm the emission is dominated by aggregates. Ability to preferentially

excite one of the AO forms offers an opportunity for selective imaging of monomeric or aggregated forms at very broad concentration range simply by changing the excitation wavelength.

AO is widely used to stain live cells where it accumulates at high concentrations in acidic intracellular compartments (typically pH 4.5–6.0) showing significant spectral shifts attributed to its aggregation [13–15]. To verify whether pH itself affects spectral properties of AO independently of its concentration we have measured emission spectra for the AO solutions of intermediate concentration of 4 mM and pH of 5.5 and 7.4, Fig. 5. With 470 nm excitation, emission spectrum shows somewhat higher content of dimers at lower pH, but overall a shift by almost 2 pH units had a relatively minor effect on the emission spectra that clearly can be attributed to slightly shifted equilibrium between monomers and aggregates.

3.3. Fluorescence lifetimes

We have analyzed time-resolved emission intensity decays for AO concentrations ranging from few μM up to saturating concentration of $\sim 105 \text{ mM}$. Two excitations wavelengths were used 435 nm and 494 nm, which preferentially excite aggregated and monomeric forms respectively, while emission was observed at the corresponding peaks of the two forms 640 nm and 530 nm respectively. The examples of intensity decays for selected concentration of 3.8 mM with 435 nm and 494 nm excitations are shown in Fig. 6 A and B respectively. For this concentration range (2–5 mM) depending on the selected excitation and emission wavelengths we can observe a short decay characteristic for a monomer, or long decay characteristic of an aggregated form. Table 1 shows details of decay parameters measured for various AO concentrations. For low concentrations below 200 μM and excitation 494 nm (monomer peak absorption) a single intensity decay of $\sim 1.75 \text{ ns}$ is observed at both 530 nm (monomer) and 640 nm (aggregate) emission wavelengths. Excitation at 435 nm (aggregate peak absorption) and observation at 530 nm indicates more complex decay, however, the average fluorescence lifetime remains very close to that observed with 494 nm excitation. Observation at 640 nm (peak aggregate emission) clearly indicates the presence of longer component already at 200 μM , but it is absent after 3–5-fold dilution producing a single exponential decay characteristic of monomeric form as presented in Table 1.

As the concentration of AO increases over 200 μM the intensity decays become more complex and fitting the data requires at least three components. For concentration of 3.8 mM the long wavelength excitation and observation at 530 nm the intensity decays show complex character, but only minor change in average fluorescence lifetimes is seen. Interestingly the average fluorescence lifetimes is slightly decreasing as concentration increases over 10 mM. This may only happen when dominant emissive species (monomers in this case) are quenched, although we are not sure about the exact mechanism of such quenching. One possible explanation could be that aggregate formation is a dynamic process in which the aggregates have significantly lower quantum yield (as measured here about 10-fold lower) and red-shifted emission. So, excited monomers are depopulated by aggregation process, while formed aggregates do not contribute to long lifetime component due to lower quantum yield and practically no emission at 530 nm. Since this process appears to be significant already at relatively low concentrations we expect that it involves formation of

dimers between an excited and ground state monomers. Also an inspection of lifetimes obtained with 435 nm excitation and 530 nm observation shows similar short amplitude average fluorescence lifetimes for higher concentration. In this case based on the absorption spectra, we expect that we preferentially excite aggregates. However, emission observed at 530 nm is probably a result of dynamic dissociation process of aggregates since the intensity decay is very heterogenic and amplitude average lifetime appears to be shorter than that of a pure monomer. We want to stress that because fluorescence lifetime of a monomer is short its diffusion is rather limited and association of two excited monomers are negligible. Also the measured decrease in average fluorescence lifetimes is smaller with 435 nm excitation (peak of aggregate excitation), which may also cause aggregates dissociation, effectively enhancing contribution of monomer emission. For long 494 nm wavelength excitation (monomer peak excitation) but 640 nm (aggregate peak emission) observation a significant long lifetime component can be observed with much longer average lifetimes already at low mM concentrations. For the concentration in the range of 3.8 mM, short 435 nm wavelength excitation shows very large change in fluorescence lifetimes indicating distinctive contribution of aggregate emission (Table 1 and Fig. 6 A and B). As AO concentrations increase above 3.8 mM longer fluorescence lifetimes become dominant reaching ~20 ns for concentrations above 30 mM.

3.4. Fluorescent labeling of mucin granules

Using epi-fluorescence microscopy and FLIM microscopy we have tested AO-staining of mucin granules in Calu-3 cells. As shown in Fig. 7A, B, AO-stained granules showed significant red-shift in fluorescence spectra and increased lifetime compared to the cytoplasm and nuclei. Based on their size distribution, Fig. 7C, and their expression in ~30–40% of the cells, they are likely mucin granules. Previous electron microscopy (EM) studies have demonstrated that 30–40% of the Calu-3 cells express apically-located granules of ~1 μ m diameter that were electron-translucent resembling mucin granules of mucous cells [3]. They have also observed similar expression level, location and size distribution with FM 1–43, quinacrine and AO staining and in immunostaining experiments with anti-MUC5AC monoclonal antibody [3].

We have also tested whether mucins have any direct effect on fluorescence spectra and fluorescence lifetime of AO. This is shown on Fig. 7D and Fig. 7E, respectively, for two different AO concentrations dissolved in PBS (pH 5.0) containing 0.05 g/ml mucin type III from porcine stomach. As expected significant red-shift in spectra and increased lifetime with increasing dye concentration between 40 μ M and 20 mM was observed, but it was not different from that shown on Figs. 3 and 6, respectively, when AO was dissolved in water and in the absence of mucins. This suggests that fluorescence spectra and lifetime are not changed in the presence of mucins at concentration used here of 0.05 g/ml, which is comparable to density of cell-secreted mucins. Thus, under these conditions spectral and lifetime changes of AO fluorescence could be solely attributed to variations in AO concentration. Whether this could be extrapolated to densely-packed mucins inside the granules remains unclear. Previous studies with parafollicular thyroid cells and mast cells attribute AO spectral changes entirely to its accumulation at high concentrations in these intracellular compartments [22,23].

For the subsequent TIRF imaging experiments we have chosen AO as a convenient fluorescent stain of Calu-3 cell granules due to easy-recognizable red-shift in fluorescence, shown in Fig. 7A. The following sections illustrate application of TIRF microscopy to study stimulated mucin secretion from AO-stained Calu-3 cells.

3.5. Real-time TIRF imaging of mucin granules secretion and swelling

Fig. 8A shows TIRF images of AO-stained cells before and ~40-s after mechanical stimulation. Mechanical stimulation consisted of gentle brief pressing of cover glass with cells facing the glass bottom window of the imaging chamber, which resulted in multiple secretion events that were seen in TIRF images as rapidly-expanding bright-fluorescence patches, indicated by arrows on the right image in Fig. 8A (see also Supplemental movie 1). Such secretory events continued for up to 10–15 min post-stimulation. An example of a time-course of a secretory event is illustrated in Fig. 8B, which shows sequential images of a zoomed-in area of Fig. 8A. It demonstrates that bright patch expansion is rapid, usually completed in <1-s and is followed by gradual fluorescence decay during the subsequent several seconds, likely due to AO diffusion out of the patch, *left graph* on Fig. 9A. After the experiment and removal of the filter membrane with cells the secretions remaining on the glass surface of the imaging chamber could be seen in DIC images, an example is shown in Fig. 8C. They formed viscous gel-like patches, as demonstrated by adherence of added glass beads. After *in situ* fixation the patches were specifically immunostained with monoclonal anti-MUC 5AC antibody, demonstrating the presence of MUC 5AC mucin.

Close inspection of images such as those shown on Fig. 8B, revealed that in addition to the secretory events described above, short fluorescence flashes of diffuse appearance and much smaller amplitude (1000–5000 AU under our experimental conditions) could also be seen. An example of such an event is shown in Fig. 9B. The intensity increase was almost instantaneous, from one frame to the next, so that its kinetics often could not be well resolved at the image acquisition rate used here (~8 frames/s). The fluorescence decayed typically in <2 s, *right graph* in Fig. 9A. Thus, the 2 types of secretory events that we observed in Calu-3 cells are characterized as: (1) bright patches with initially well-defined edges, high peak fluorescence intensity, occasionally reaching saturating level of the image detector (~65,000 AU), and prolonged decline of fluorescence of ~10 s; and (2) brief <2 s flashes of diffuse appearance, and smaller amplitude typically <5000 AU under our experimental conditions. We interpret these two types of events as originating from exocytosis of two different populations of vesicles: (1) loaded with high density cargo, mucin-containing granules, and (2) loaded with low-density cargo. This notion is further supported by the analysis of the kinetics of cargo release/expansion and different diffusion coefficients of AO after its exocytotic release from the 2 types of vesicles (see below).

Kinetics of mucin patch expansion is further analyzed in more details in Fig. 10 A, B. It shows three examples of post-secretory mucin patch area changes for patches of different final (maximal) size, indicated (in μm^2) on each graph. The patch area was determined from the number of pixels with fluorescence intensity exceeded a fixed threshold level set for the given experiment, see Methods. In this approach, after initial rapid swelling, the apparent patch size decreased due to diffusion of AO out of the mucus patch and decay of

fluorescence signal. The swelling phase is analyzed in more details on the lower graphs of Fig. 10A, which shows that the patch radius increase could be well fitted with the first order kinetics equation (see Methods). For the 3 examples shown (final mucin patch area of 46-, 26.1-, and 13.4- μm^2) the corresponding time constants (in milliseconds) were 389 ± 17 , 238 ± 16 , and 161 ± 39 . Data demonstrate that the smaller the final patch size, the faster was the swelling. Fig. 10B summarizes data from several experiments and shows that the time constant τ increases linearly with the square of the final radius r_1 , as expected for first-order kinetics of mucin gel swelling. The slope of this linear relationship $\tau = r_1^2/D$ corresponds to D of $3.98 \pm 0.07 \times 10^{-7} \text{ cm}^2/\text{s}$. This value agrees quite well with diffusivity of mucus from normal human goblet cells $3.1 \pm 1.2 \times 10^{-7} \text{ cm}^2/\text{s}$ reported by Verdugo [6] that was investigated by phase-contrast video microscopy under similar experimental conditions, i.e., in extracellular media containing 1 mM CaCl_2 .

3.6. AO shows restricted diffusion in the mucin patch

In our experimental configuration mucin granule content is secreted into a confined extracellular space between the cell plasma membrane and the glass substrate, likely remaining there for the rest of the experiment. After secretion, however, AO is free to diffuse out of the mucus patch since it is not bound to the mucin macromolecules. The resulting fluorescence decay could provide information on AO diffusibility in the mucin gel. Fig. 11A shows an example image of a mucin patch at its maximal expansion, and below are shown sequential images of a zoomed-in area showing fluorescence decay over time. The graph in Fig. 11B shows fluorescence intensity profiles along a line crossing through the center of the patch (indicated on upper image in A) observed at different time points after secretion. This linescan shows well-defined intensity peak whose height was decreasing over time. The intensity peaks could be well fitted with a Gaussian distribution and an example of such fit is shown on Fig. 11C. The width of the peak is directly related to the diffusion coefficient of AO in the mucus patch and the time elapsed after the secretion. For the example shown, the diffusion coefficient was $3.52 \times 10^{-7} [\text{cm}^2/\text{s}]$, but it varied somewhat between different mucus patches from 1.7×10^{-7} to $4.29 \times 10^{-7} [\text{cm}^2/\text{s}]$. These values are approximately one order of magnitude smaller compared to AO diffusion coefficient in bulk water of $4.2 \pm 0.5 \times 10^{-6} [\text{cm}^2/\text{s}]$ [24], consistent with its diffusion in a dense mucus gel. Similar analysis of AO diffusion after exocytotic release of low density vesicles (type 2 secretory events) is illustrated on Fig. 12. These secretory events are of lower intensity, shorter duration and wider intensity profiles compared to those for mucin patches illustrated above on Fig. 11. The corresponding diffusion coefficients calculated from the Gaussian fit to the intensity profiles were $1.99 \pm 0.77 \times 10^{-6} [\text{cm}^2/\text{s}]$. This is only ~2-fold smaller than AO diffusion in bulk water [24], clearly demonstrating that the cargo released in type 2 events is of low density, in contrast to dense mucin gel which shows ~10 times lower D illustrated on Fig. 11.

3.7. Advantages and limitations of the TIRF assay and AO fluorescence staining

3.7.1. TIRF assay—Our study demonstrates that TIRF microscopy assay allows quantitative characterization of single mucin granule exocytosis and determination of post-exocytotic swelling kinetics of the released content. The swelling kinetics provides important information on the diffusivity of mucus matrix in a defined extracellular solution.

Here, we used Calu-3 cells and observed exocytosis and rapid swelling of AO-stained, high density granules with corresponding diffusivity in the order of 10^{-7} [cm^2/s], which is expected for diffusion of small solutes into the mucin gel. In addition, post-secretory fluorescence decay permits estimates of AO diffusivity in the secreted mucus offering complementary information on its microrheological properties. Such capabilities enabled us not only to detect secretory events involving mucin granules, but also to distinguish them from exocytosis of vesicles containing low density load. The latter one were of smaller amplitude and showed much faster post-secretory fluorescence decay, consistent with their smaller AO content and less restricted diffusion similar to that in aqueous environment. Since composition of extracellular solution in this assay could be easily manipulated, effects of varying pH and ionic composition, e.g. concentration of Ca^{2+} and HCO_3^- , on mucin swelling kinetics and its rheological properties, could be tested. Together with the use of normal and CF mucus secreting cells, as well as use of CFTR channel function blockers, such studies could provide new important insights into the role of CFTR in mucus abnormalities observed in CF [11].

Potential limitations of the method described here may arise from the fact that mucin secretion and swelling takes place in the narrow space between the glass surface of the imaging chamber and the closely positioned cell plasma membrane. The exact thickness of this space is difficult to estimate, but because fluorescent vesicles were often visible in several areas in TIRF images, it suggests that in these areas it was comparable to the evanescent field depth of ~ 100 nm. There are two consequences of such geometrical configuration, first is the difficulty to estimate absolute volume of secreted mucin patch, since only its thin layer adjacent to the glass substrate is illuminated and visualized in TIRF microscopy. Secondly, such configuration may potentially impact the kinetics of swelling/hydration by limiting diffusion of H_2O and solutes into the mucin gel. Interestingly however, the experimental D values obtained here are in excellent agreement with those reported for mucin swelling in bulk solution investigated with phase contrast video microscopy [6], suggesting that accessibility of solutes and water to the swelling mucin patch is not a rate limiting factor. A possible explanation for this observation may involve a fact that rapidly swelling mucin gel may locally push and lift the plasma membrane of a soft cell away from the rigid glass surface to accommodate its increasing volume while facilitating proper hydration. Verification of such a mechanism would require simultaneous imaging of fluorescently stained plasma membrane and mucin patch during the secretion process.

3.7.2. AO as a fluorescent dye—Confocal FLIM images show that AO accumulates in mucin granules at high concentration exhibiting red long lived emission. In the present study we utilized a single-line TIRF imaging and measured intensity of AO-stained granules and mucins during post-secretory swelling. However, the unique AO properties described in this study open a possibility for selective excitation and observation of AO monomers and aggregates. An advantage of selective visualization of different AO forms is that it will significantly increase dynamic range and sensitivity of their detection. In addition, two-color TIRF excitation and two wavelengths observation should enable more quantitative ratiometric imaging of the two forms significantly speeding-up image acquisition process.

Such imaging advances could facilitate investigations of AO concentration gradients (and/or heterogeneities) within single-granule mucin patch, likely reflecting mucin density gradients. Detailed insights into the dynamics of a multistep mucin swelling process may help to understand abnormalities of mucus rheology in CF and other diseases.

3.8. Conclusions and future directions

Presented studies definitely prove that TIRF-based assay allows visualization and quantitative analysis of post-secretory mucin swelling kinetics in live cells with high sensitivity of detection and high spatial and temporal resolution. Current approach was limited to a single wavelength excitation and long wavelength observation that allowed us collecting images with high rate of over 10 frames per second. As such speed is relatively easy to achieve with intensity imaging, collecting time-resolved images for FLIM at such high speed would be a significant challenge with widely available detectors. Our time-resolved studies of AO fluorescence demonstrated that using two excitations wavelengths 435 nm and 494 nm, and two observation channels 520–540 nm bandwidth and 640 nm long pass, will greatly increase dynamic range and sensitivity of detection. More importantly, such two-color excitation can be achieved in so called interleave excitation mode with two detectors tuned to a specific wavelength. A simple ratiometric signal from the two detectors could reveal time-dependent information of AO concentration distribution (monomer–dimer equilibrium) within a sample. Such ratiometric imaging could be accomplished with high speed using currently available detectors, while the information content will be comparable to that of FLIM analysis. Obviously with the development of faster detectors a high-speed FLIM analysis should be warranted to further increase the sensitivity and dynamic range. In summary, the live-cell imaging assay described here, even in its present single-line TIRF format, should be highly valuable research tool with potentially many biological applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ymeth.2013.09.004>.

Fig. 1.

The concept for TIRF detection of mucin granule exocytosis. A cartoon shows a cell placed in “upside-down” orientation on the glass bottom of an imaging chamber observed with an inverted fluorescence microscope (objects are drawn not to scale). In this orientation the cell’s apical membrane is positioned in close proximity of the evanescent field extending from the glass substrate. Fluorescently-labeled mucin granules will be visible in the through-the-objective total internal reflection (TIR) illumination only when they reach the evanescent field near the plasma membrane. Upon exocytotic secretion mucin fluorescence will dramatically increase since evanescent field intensity increases exponentially towards the glass substrate. The post-exocytotic mucin swelling will produce a rapidly expanding bright-fluorescent patch.

Fig. 2. (A). Normalized absorption spectra of AO at various concentrations. (B). Ratio of normalized absorption spectra of the highest AO concentration (105 mM, AO aggregates) to the lowest concentration (9.5 μ M, AO monomers). The ratio reflects relative contributions of both AO forms to total absorption. Excitation at 435 nm will preferentially excite aggregates while 494 nm will preferentially excite monomers.

Fig. 3. Normalized emission spectra of AO water solution at concentrations from 10 μ M to 100 mM observed with 494 nm excitation wavelength (A), and 435 nm excitation (B).

Fig. 4.

Normalized emission spectra measured for two AO concentrations of 2 mM (A) and 7.5 mM (B), with excitation wavelengths of 435 nm and 494 nm respectively. For 2 mM AO and 494 nm excitation monomer emission dominates, while 435 nm excitation produces almost equal emissions of monomer and aggregate fractions (A). For higher concentration of 7.5 mM the opposite is observed, at 435 nm excitation AO aggregate emission dominates, while 494 nm excitation produces equal emissions from both AO fractions (B).

Fig. 5. Normalized emission spectra for AO at 3.8 mM concentration measured at pH 5.5 and pH 7.4 with 470 nm excitation wavelength from laser diode. A shift of almost 2 pH units had a relatively minor effect on the emission spectra.

Fig. 6. Example of fluorescence intensity decays of 3.8 mM AO solution. (A) Intensity decay observed at 530 nm and 640 nm with 435 nm excitation. (B) Intensity decay measured at 530 nm and 640 nm with 494 nm excitation. Both excitation wavelengths were selected from Fianium (super-continuum) laser system through a prism monochromator.

Fig. 7.

Fluorescent labeling of Calu-3 cells mucin granules with acridine orange. (A, B): Epi-fluorescence and FLIM images of AO-stained Calu-3 cells. Images show red-shift of the fluorescence (from green to orange) and longer lifetimes in the granules compared to cytoplasm or nucleus. Images were acquired with fluorescence Zeiss Axioobserver microscope (A), and confocal Olympus IX71 microscope equipped with a PicoQuant fluorescence lifetime system (B). (C): Size distribution of AO-stained Calu-3 granules determined from epi-fluorescence images such as that shown in (A). (D, E): Fluorescence spectra and fluorescence life time respectively of AO dissolved in PBS (pH 5) containing 0.05 mg/ml mucin type III from porcine stomach is shown for dye concentration of 38 μM and 18.18 mM (green and red lines respectively), blue trace shows instrument response function IRF. Increased dye concentration produced significant red-shift of fluorescence maximum from 525-nm to ~650-nm, and significant increase of fluorescence life time (from 4.1-ns to ~12-ns), but it was not different in the presence or absence of up to 0.05 mg/ml of type III mucins from porcine stomach (not shown). Fluorescence spectra were measured

with Cary Eclipse spectrofluorometer (Varian Inc., Australia). For lifetime measurements, FluoTime 200 (PicoQuant GmbH, Germany) time domain spectrofluorometer was used.

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Fig. 8.

TIRF imaging of mucin granules secretion. (A) TIRF images (280×280 pixels) of AO-stained cells before and ~40-s after mechanical stimulation, left and right images respectively. Arrows point to rapidly-expanding bright-fluorescence patches that were seen after mechanical stimulation, right image. (B) An example of a time-course of mucin granule exocytosis and post-exocytotic mucin swelling. The panel shows sequential images (100×100 pixels) of a zoomed-in area of Fig. 8A, frame numbers are indicated in the lower-left corner of each image. The images were acquired at ~120-ms intervals, exposure

time 30-ms. C, immunostaining of secreted mucins. Left picture is a DIC transmitted light image of translucent gel-like secretions deposited on the chamber glass bottom after an experiment such as shown in A, B. The glass beads (diameter $<100\text{ }\mu\text{m}$) added to the chamber remained trapped by sticky, viscous patches of secreted mucins. Middle picture shows fluorescence image of the same sample, which was immunostained with Alexa 488-labelled IgG antibody conjugated with anti-Mucin 5AC monoclonal antibody; right image is an overlay of DIC and fluorescence images. The images are 508×504 pixels ($812 \times 806\text{ }\mu\text{m}$).

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Fig. 9.

Time-course of fluorescence intensity changes after stimulated secretion. (A) *left graph*, an example of mucin patch average intensity changes as seen by TIRF microscopy during exocytotic release. The rising phase represents post-exocytotic mucin swelling and the following decay of fluorescence is due to AO diffusion out of mucin patch. In this example another exocytotic event in the neighboring, partially overlapping area (ROI), occurred at the time of peak response producing apparent biphasic fluorescence decay. (B) Example of a time-course of low intensity exocytotic AO release event. The panel shows sequential images (50×50 pixels) with frame number and time indicated under each image. Note diffuse appearance and short duration of the event compared to that of Fig. 8B. Its average intensity time-course is shown in A, *right graph*.

Fig. 10.

Swelling kinetic of mucin patch. (A) *Upper graphs*, show three examples of post-secretory mucin patch area changes, their maximal size after expansion is indicated (in μm^2) on each graph. The 3 *lower graphs* show the corresponding time-course of mucin patch radius increase during the swelling phase. The data points could be well fitted with a single exponential function (red line) and the corresponding time constant τ (in seconds) is indicated on each graph. (B) Characteristic time of patch expansion increases linearly with square radius as expected for gel swelling. The slope of the linear relationship $\tau = r_1^2/D$ corresponds to D of $3.98 \pm 0.07 \times 10^{-7} \text{ cm}^2/\text{s}$.

Fig. 11.

Diffusion of AO in the secreted mucin patch. (A) An example image of mucin patch at its peak expansion, frame 450. Below are shown zoomed images (50×50 pixel) of the same patch illustrating a time course of fluorescence intensity decay. Frame #, acquisition time and time elapsed from the start of secretion (in seconds) are indicated below each image. (B) Intensity profiles along a line (shown in A), crossing the patch. The profiles are shown at different time points (indicated by frame #) after secretion, the secretion event started at frame 442, ($t = 56,529$ s), not shown. C, An example of a GAUSSIAN fit to the linescan

intensity profile. The black squares represent the data points for the image of frame #475 shown in B, while the red solid line is a GAUSSIAN fit to the data.

Fig. 12.

Diffusion of AO after exocytotic release of low density vesicles. (A) An example of exocytotic cargo release from low density vesicle. The sequence of images (20×20 pixels) illustrates the time-course of release. Frame #, acquisition time and time elapsed from start of secretion (in seconds) are indicated below each image. (B) Intensity profiles (linescans) along a line crossing the center of the images shown in A, at different time points after secretion. The secretion event started at frame 889, ($t = 116.689$ s), shown in Fig. 9(B). (C) An example of a GAUSSIAN fit to the linescan intensity profile. The black squares represent the data points for the image of frame #892 shown in B, while the red solid line is a GAUSSIAN fit to the data.

Table 1
Recovered parameters for time-resolved intensity decays for 494 nm and 435 nm excitations and 640 nm and 530 nm observations.

	Concentration mM	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	α_1	α_2	α_3	τ	χ^2
Exc = 494 nm	<0.2	1.75	–	–	1	–	–	1.75	1.75
Obs = 530 nm	3.8	2.431	1.547	0.504	0.07	0.905	0.025	1.53	1.48
	15	6.542	1.251	0.396	0.011	0.906	0.083	1.23	1.07
	52	12.881	0.873	0.302	0.094	0.674	0.232	1.87	0.65
	105	18.118	1.204	0.313	0.363	0.103	0.534	6.87	0.54
Exc = 494 nm	<0.2	1.76	–	–	1	–	–	1.76	1.76
Obs = 640 nm	3.8	13.914	5.701	1.492	0.541	0.166	0.293	8.90	3.80
	15	17.615	8.922	1.262	0.720	0.220	0.060	14.72	8.78
	52	20.603	8.305	1.004	0.870	0.110	0.020	18.86	13.72
	105	21.172	7.702	1.408	0.861	0.121	0.018	19.19	14.48
Exc = 435 nm	<0.2	1.779	0.219	–	0.989	0.011	–	1.76	1.64
Obs = 530 nm	3.8	2.434	1.567	0.476	0.026	0.938	0.036	1.64	1.47
	15	13.082	1.280	0.409	0.098	0.807	0.094	2.36	1.15
	52	18.089	1.076	0.360	0.497	0.307	0.196	9.38	1.16
	105	19.593	2.398	0.255	0.786	0.087	0.127	15.64	1.74
Exc = 435 nm	<0.04	1.78	–	–	1	–	–	1.78	1.78
Obs = 640 nm	0.2	5.721	1.831	–	0.077	0.923	–	2.64	2.13
	3.8	16.106	9.681	1.765	0.452	0.440	0.108	11.73	7.42
	15	18.571	10.837	1.546	0.680	0.294	0.026	15.86	12.43
	52	20.884	8.518	1.178	0.885	0.102	0.013	19.36	15.26
	105	21.029	6.534	1.230	0.890	0.098	0.012	19.36	14.81