Utilization of integrated correlative light and electron microscopy (iCLEM) for imaging sedimentary organic matter

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Summary

We report here a new microscopic technique for imaging and identifying sedimentary organic matter in geologic materials that combines inverted fluorescence microscopy with scanning electron microscopy and allows for sequential imaging of the same region of interest without transferring the sample between instruments. This integrated correlative light and electron microscopy technique is demonstrated with observations from an immature lacustrine oil shale from the Eocene Green River Mahogany Zone and mid-oil window paralic shale from the Upper Cretaceous Tuscaloosa Group. This technique has the potential to allow for identification and characterization of organic matter in shale hydrocarbon reservoirs that is not possible using either light or electron microscopy alone, and may be applied to understanding the organic matter type and thermal regime in which organic nanoporosity forms, thereby reducing uncertainty in the estimation of undiscovered hydrocarbon resources.

Introduction

The development of hydrocarbon production from shale reservoirs in North America over the last decade has revitalized the use of scanning electron microscopy (SEM) to characterize nanometre-scale textures in ion-milled sample preparations. Investigators are particularly concerned with the interconnected nanoporosity contained in shale organic matter (Loucks et al., 2009; Loucks et al., 2012) that is thought to form the primary hydrocarbon migration pathway (permeability) and storage reservoir (porosity) (Löhr et al., 2015). Although organic matter is easily identified in SEM by its characteristic low backscatter electron (BSE) intensity (appearing dark grey in standard greyscale images), SEM is poorly suited to identify the individual organic matter types, e.g. kerogen versus solid bitumen, which are easily recognized by standard optical microscopy techniques (Hackley & Cardott, 2016). The traditional organic petrography approaches such as fluorescence microscopy and incident white light observation under oil immersion can provide conclusive identification of organic matter. However, these optical techniques are limited by relatively low magnifications (up to about 1500×) compared with the high-magnification capabilities of SEM (up to >100 000×). Some SEM practitioners have employed general morphological and textural ‘rules of thumb’ for organic matter identification in SEM (Loucks & Reed, 2014; Camp, 2016), but these approaches often are inconclusive. Because of this limitation, SEM, while invaluable for high-magnification shale reservoir characterization and detection of organic nanoporosity, cannot conclusively identify the organic matter type in which porosity has developed or the thermal regime at which it forms.

Several studies have employed correlative microscopy techniques to identify and characterize shale organic matter and its porosity (Bernard et al., 2012; Fishman et al., 2012; Baruch et al., 2015; Cardott et al., 2015; Löhr et al., 2015; Camp, 2016; Luo et al., 2016). However, these studies utilized multiple instruments, which necessitates time-consuming sample mapping, sample transfer between instruments and relocation of the regions of interest (Timmermans & Otto, 2015). In addition, this approach often involves collaboration between multiple laboratories and analysts, thereby increasing difficulties for field relocation, reorientation and the likelihood for sample damage during transfer.

Correlative light and electron microscopy (CLEM) techniques are widely applied in cellular biology (Polishchuk et al., 2000; Sartori et al., 2007; de Boer et al., 2015). Integrated CLEM (iCLEM) techniques, where both imaging modalities are performed sequentially in the same instrument without sample transfer, have also been used in biology and biomedical...
Fig. 1. Correlative light and electron microscopy images of the same field of a low-maturity organic-rich Bakken Shale sample from North Dakota, USA. (A) Secondary electron (SE) SEM image showing organic matter (dark grey to black). Greyscale contrast is evident on the high reflectance inertinite (red arrow in all four images) due to differences in relief. (B) Backscatter electron (BSE) SEM image showing little contrast in dark grey to black organic matter. (A) and (B) collected using 5 kV, 10 SI, 5 mm WD. (C) White incident light oil immersion image showing differences in reflectance of the two inertinites, solid bitumen and amorphous organic matter of algal origin (Stasiuk & Fowler, 2004). (D) Blue light (BL) fluorescence image showing absence of autofluorescence in inertinite and solid bitumen and its presence in the amorphous organic matter. Blue light fluorescence imaging used 445 nm LED excitation with a 482 nm beam splitter and 510 nm long-pass emission filter combination. From Valentine & Hackley (2016). Correlative imaging was performed using sample transfer between two separate microscope systems: Leica DM-4000 for light microscopy and Hitachi SU-5000 FE-SEM for electron microscopy. $R_r =$ random reflectance. See also http://energy.usgs.gov/Coal/OrganicPetrology/PhotomicrographAtlas/BakkenFormationShaleGallery.aspx.

imaging (Agronskaia et al., 2008; Faas et al., 2013; Zonnevylle et al., 2013; Vidavsky et al., 2014; Brama et al., 2015; de Boer et al., 2015, among others). We describe, to our knowledge, the first application of iCLEM for geological materials, utilizing simultaneous fluorescence and SEM to image sedimentary organic matter in shale, including an immature oil shale from the Eocene Green River Mahogany Zone, Colorado, USA, and from mid-oil window paralic shale of the Upper Cretaceous Tuscaloosa Group, southern Mississippi, USA. Selection of these samples was influenced by a recent correlative microscopy study (Valentine & Hackley, 2016) which illustrated utility of light microscopy versus SEM for differentiation of organic matter types at low thermal maturities [0.32% solid bitumen reflectance ($BR_o$)]. In particular, SEM imaging of a $\sim 60 \times 75 \mu m$ field in immature Bakken Shale via secondary electron (Fig. 1A) and BSE (Fig. 1B) showed little or no contrast between various forms of organic matter that are easily distinguished with reflected white light (Fig. 1C) and fluorescence microscopy (Fig. 1D). Based on the results of this initial study, we tested the capabilities of iCLEM imaging for identification and differentiation of organic matter types in the Green River Mahogany Zone and Tuscaloosa Group samples.

Methods

Samples

We used an organic-rich [22 wt.% total organic carbon (TOC)] lacustrine oil shale from the Eocene Green River Mahogany Zone in Colorado, USA. Previous studies (Hackley et al., 2015) characterized the dominant organic matter in this sample as strongly fluorescent amorphous and lamellar type I kerogen interpreted as a benthic microbial mat deposit (Fig. 2). Scattered types III and IV kerogen (vitrinite and inertinite) and lamellae of solid bitumen (a solid hydrocarbon) are also present. Vitrinite reflectance ($VR_o$) of the Green River sample is
**Fig. 2.** Conventional light microscopy of Eocene Green River Mahogany Zone lacustrine oil shale (collected from Anvil Points Mine, Rifle, Colorado). (A) Histogram of mean vitrinite reflectance measurements reported from multiple laboratories (Hackley et al., 2015). (B) Fluorescence spectrum (average of 10 measurements) of amorphous organic matter showing peak fluorescence intensity at ~555 nm. Spectra were corrected to a common illumination source according to specific microscope conditions (Baranger et al., 1991). (C) Blue light fluorescence image of example amorphous organic matter from which fluorescence spectra were collected (see filter settings in the caption of Fig. 1). (D) Image from flatbed scanner of whole thin section showing alternating organic-rich (darker) versus carbonate-rich (lighter) lamellae. (E) Image mosaic of whole thin section illuminated with blue light fluorescence (from high brightness 445 nm blue LED). (F) White incident light oil immersion photomicrograph showing grey solid bitumen lamellae and low-contrast amorphous organic matter (AOM). (G) Same field as F under blue light fluorescence (see filter settings in the caption of Fig. 1).
Fig. 3. Thin section images and oil immersion photomicrographs of organic matter in the organic-rich (~7 wt.% TOC) oil window maturity (~0.75–0.80% \( R_o \)) lower Tuscaloosa shale sample. This sample is from the Samedan No. 2 C.W. Andrews well (API 23005203530000) at 11 068 ft (3374 m), Amite County, southern Mississippi. (A) Image from flatbed scanner of whole thin section. (B) Image mosaic of whole thin section illuminated with blue light fluorescence. (C) Transmitted plane-polarized light. (D) Same field as C under blue light fluorescence. (E) Same field as (C) and (D) in white incident light. (F) Transmitted plane-polarized light. (G) Same field as (F) under blue light fluorescence. (H) Same field as (F) and (G) in white incident light. Modified from Hackley & Cardott (2016).
0.31%, indicating that conditions are immature for hydrocarbon generation (Hackley et al., 2015). The second sample was an organic-rich (7 wt.% TOC) paralic shale from the Upper Cretaceous Tuscaloosa Group (lower Tuscaloosa Formation) of southern Mississippi, USA. Previous work (Hackley & Cardott, 2016) described the organic matter as a brightly fluorescent groundmass with lamellar algal (?) material, fluorescent solid bitumen and subordinate types III and IV kerogens vitrinite and inertinite (Fig. 3). VRo, for the Tuscaloosa sample likely is 0.75–0.80% based on data from a nearby well, indicating the sample is within the oil-generating window.

**Sample preparation**

Two sample preparation methods were tested. For both approaches, samples were prepared on glass slides (about 1.2 mm thickness) as standard uncovered, polished rock thin sections (Rowland, 1953), ground to a final thickness of approximately 20–30 µm. Cyanoacrylate (Super-Glue™) was used as adhesive to attach the rock sections to the glass slides. Final polish used a 0.25 µm abrasive. In the first approach, the section was removed from the slide via dissolution of the cyanoacrylate adhesive in acetone and transferred to a 170 µm thickness 14 × 14 mm glass cover slip for iCLEM imaging. This approach was found to be unsatisfactory for multiple reasons, as described below.

The second approach employed wedging of the thin section, i.e. intentionally creating a section of uneven thickness by polishing one side to a vanishing edge (Fig. 4). Wedging is accomplished by simply applying uneven pressure with finger tips during final polish of the thin section. The wedged thin section was then scored and broken to fit (<1 in: 25.4 mm)
Fig. 5. (A) Photograph of SECOM iCLEM system. (B) Installation of SECOM on FEI Quanta SEM. (C) Schematic illustrating configuration of light and electron microscopy features of SECOM iCLEM system.

We used the Delmic SECOM platform (Fig. 5A) installed on an FEI Verios 460 L scanning electron microscope (Zonnevylle et al., 2013) to image the immature oil shale and the paralic shale. The SECOM instrument configuration allows simultaneous top-down SEM using both backscatter and/or secondary electron detectors, and bottom-up fluorescence imaging with the sample positioned in the middle (Fig. 5B). Fluorescence imaging used a four-colour LED light source and scCMOS camera and a quad-band filter cube with excitation maxima at 392, 474, 554 and 635 nm. For the initial sample preparation approach, the separated rock thin section transferred to a glass cover slip was imaged with a 40×/0.95 NA plan apochromatic fluorescence objective (diffraction limit about 290 nm @ 555 nm). The standard thickness (20–30 μm) uncovered thin section of the Tuscaloosa shale was also imaged using a 40×/0.60 NA extra-long working distance objective (diffraction limit about 460 nm @ 555 nm) without transfer from the glass slide.

For the second sample preparation approach, the Ar ion-milled broken piece of rock thin section from the Mahogany Zone was placed directly into the SECOM. The 40×/0.60 NA
Fig. 6. Integrated CLEM (iCLEM) images of organic-rich, mid-oil window Tuscaloosa Group paralic shale. (A) 100% fluorescence (FM), 0% SEM (EM). (B) 60% fluorescence, 40% SEM. (C) 40% fluorescence, 60% SEM. (D) 100% SEM. (E) 40% fluorescence, 60% SEM of region of interest shown by red rectangle in (D). Note misregistration of fluorescence (green arrows) with dark organic matter layers imaged by SEM (red arrows and red layer margins) and polishing relief between organic and inorganic layers. Fluorescence imaging used a four excitation LED Pinkel configuration with excitation at 392, 474, 554 and 635 nm and multiband beam splitter and emission filters. Note that false colour fluorescence in (A) includes red and blue not seen in traditional epifluorescence. SEM imaging at 10 kV using the Everhart-Thornley detector (ETD) for secondary electron contrast.
Fig. 7. iCLEM images of organic-rich immature Mahogany Zone lacustrine oil shale. (A) Wide-field false colour fluorescence image with 50:50 SEM region of interest (ROI) overlay. (B) 50:50 zoom-in to area at margin of SEM ROI. (C) 80:20 view of zoom-in area. (D) 100% SEM view of zoom-in area. Red arrows point to examples of authigenic carbonate visible only in SEM. False colour fluorescence created by combination of 474 and 554 nm excitation. Cathodoluminescence shown in red. SEM imaging at 20 kV using the configurable backscatter detector (CBS).

ELWD objective was used for fluorescence imaging through the 170 μm thickness glass slide. For data presented in Figure 6, the SEM was operated in secondary electron mode at 10 kV. SEM images shown in Figures 7–9 were acquired at 20 kV using a configurable backscatter detector (CBS).

To examine the Mahogany Zone sample, we used the iCLEM microscope to detect cathodoluminescence. A selected region of interest (ROI) was continuously scanned with the electron beam while simultaneously detecting the cathodoluminescence using the sCMOS camera of the light microscope. This was done with the quad-band filter in place which reduced the observed cathodoluminescence by about half of the generated light intensity.

Results and discussion

Standard thin section

We found problems with the initial approach due to transfer of the thin section from the glass slide to the glass cover slip. Because of the fragile nature of the thin section, bumps and folds were created during transfer, causing focus issues related to nonflatness of the section. Difficulties also were encountered with poor adhesion of the thin section to the glass cover slip, resulting in unintended movement of the section during vacuum draw-down and realignment of the stage in the SEM. This approach was deemed unsuccessful and no imaging results are presented here.

By replacing the 40×/0.95 NA fluorescence objective with a 40×/0.60 NA long working distance objective, there was no need to remove the section from the glass slide and transfer it to the cover slip. Instead, fluorescence imaging was possible through the glass slide, although with reduced resolution. In Figure 6, we show results from correlative imaging of the sample from the Tuscaloosa shale. Although this approach produced usable iCLEM results, the sample thickness of 20–30 μm caused misregistration to occur between fluorescence emission from the lower side of the sample compared to electron observation from the upper side. Correlative images (A)–(D) in Figure 6 show a transition from 100% fluorescence to 100% SEM observation, whereas Figure 6(E)
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Fig. 8. iCLEM images from Mahogany Zone sample. (A) 60% fluorescence microscopy, 40% SEM overlay showing solid bitumen lamellae adjacent to amorphous organic matter-rich layer. (B) 30:70 FM to SEM view of zoom-in region in (A). (C) Same field as (B) with 100% SEM view. (D) Zoom-in to ROI shown in (C) with 30:70 FM to SEM view. See the caption of Figure 7 for imaging conditions.

shows the indicated ROI from Figure 6(D) at higher magnification. In Figure 6(E), we have highlighted a dark (in SE-SEM view) organic matter-rich layer in red (red arrows), whereas corresponding fluorescence from the lower side of the section is indicated with green arrows. There is clear misregistration of the fluorescence response in both placement and thickness in comparison to the SEM imaging. This is due to 10s of µm separation (because of sample thickness) between the two imaged areas. In addition to misregistration, residual scratches from the 0.25 µm final polish were observable via SEM (not shown) and high relief occurs between organic and inorganic phases due to polishing hardness. Nevertheless, despite that iCLEM imaging of a standard uncovered thin section preparation showed misregistration in this initial study, we have included its description because of the advanced thermal maturity of the imaged sample. The Tuscaloosa Group sample was collected from the mid-oil window of thermal maturity \( [\text{vitrinite reflectance (VR}_o] \approx 0.75–0.80\% ] \) in an area where self-sourced hydrocarbons are commercially produced from overlying strata (Lu et al., 2015). Although organic porosity was not observed in the Tuscaloosa sample, it is thought to develop in kerogen and solid bitumen as a consequence of thermal advance through the oil window due to the expulsion of generated hydrocarbons (Löhr et al., 2015). Therefore, successful fluorescence imaging of this thermally mature sample demonstrates that iCLEM imaging via the SECOM platform can be applied up to at least mid-oil window conditions, potentially helping to constrain the thermal regime and organic matter types in which organic porosity develops.

Argon ion-milled thin section

Using the Ar ion-milled thin section resulted in improved alignment between fluorescence emission and SEM imaging as well as lower relief and fewer residual artefacts from sample preparation on the milled surface. In Figure 7(A), an SEM ROI (grey square) is shown in the centre of a larger field imaged by false colour fluorescence microscopy in the Green River sample. The SEM image overlays the fluorescence image in a 50:50 viewing ratio in Figure 7(A). In Figure 7(B), a smaller ROI (red rectangle in Fig. 7A) is selected at the margin of the SEM ROI, still with the 50:50 viewing ratio. Apparent in the 50:50 viewing area are authigenic micron-scale carbonate minerals (red arrows, right side of Fig. 7B) which are not visible in the false colour fluorescence area due to the high fluorescence intensity signal from the amorphous organic matter (AOM) (left side of Fig. 7B). Note a solid bitumen lamella which is distinguished from AOM by its lack of fluorescence. The lamella is visible in the 50:50 view of Figure 7(B) and also in the 80:20 view of Figure 7(C). However, the solid bitumen lamella is indistinguishable from adjacent fluorescent AOM in the 100% SEM image (right side, Fig. 7D).

The utility of iCLEM is further expanded in the images of Figure 8 where we show a second ROI, also with two types
Fig. 9. iCLEM images of Mahogany Zone carbonate concretion or microbialite margin showing cathodoluminescence response (in pinkish-red). (A) Wide-field false colour fluorescence image with 50:50 FM to SEM region of interest (ROI) overlay with cathodoluminescence detection area shown by red rectangle. (B) 80% fluorescence:20% SEM-cathodoluminescence zoom-in. (C) 50:50 view of zoom-in area. (D) 100% SEM view of zoom-in area. (E) Further zoom-in showing 70% fluorescence:30% SEM. Red arrows point to nonluminescent euhedral crystal overgrowths embaying strong fluorescence intensity amorphous organic matter, whereas dark blue arrow points to nonfluorescent solid hydrocarbon embayed by euhedral terminations. (F) Same field as (E) with 100% SEM view. See the caption of Figure 7 for imaging conditions.
of organic matter. Figure 8(A) shows a wide field of view including the SEM ROI (light grey square, upper left), whereas Figure 8(B) shows an enlarged view of the area marked by the red rectangle in Figure 8(A) at a 30:70 display ratio. Two different types of organic matter are visible in Figure 8(B): nonfluorescent solid bitumen adjacent to strongly fluorescent AOM. However, these organic matter types are indistinguishable in the 100% SEM image of Figure 8(C), due to the lack of greyscale contrast. Figure 8(D) shows an enlargement of the ROI marked by the red rectangle in Figure 8(C), where combined SEM and fluorescence images resolve submicron carbonate and the two organic matter types are differentiated on the basis of fluorescence. The pinkish-red in the carbonate shown in Figures 8(A), (B) and (D) is from cathodoluminescence. Euhedral carbonate embays solid bitumen (right side, Fig. 8D). As expected due to the low thermal maturity (0.31% R₀), no organic porosity was resolved in either the solid bitumen or amorphous kerogen observed in our study. However, we primarily focused on low-magnification simultaneous fluorescence-SEM imaging, whereas 25 000–100 000 × magnifications typically are used in studies of organic nanoporosity (Loucks et al., 2012). The iCLEM SECOM platform, which can be retrofit to any SEM instrument, does not prohibit these high magnifications. Because SEM on its own cannot conclusively identify organic matter type, the ability of fluorescence microscopy in iCLEM to differentiate nonfluorescent and fluorescent organic matter types (in this case solid bitumen and adjacent AOM) at micron-scale resolution, will be critical to studies which attempt to characterize the thermal regime in which organic nanoporosity develops and the organic matter types that it forms in. Once organic matter type identification is confirmed at micron scale, the SEM side of the iCLEM configuration can then be used to evaluate organic porosity at nanoscale.

Cathodoluminescence is used in silicate-rich settings (Milliken et al., 2012) and carbonate systems such as the Mahogany Zone to distinguish detrital from authigenic minerals for better understanding of diagenesis and to study porosity evolution (Boggs & Krinsley, 2006; Hiatt & Pufahl, 2014). Full discussion of diagenesis in the Mahogany Zone is beyond the scope of this paper but we emphasize that cathodoluminescence from iCLEM imaging of the Green River sample reveals organic–inorganic diagenetic interactions that may not otherwise be as apparent using a single microscope application. Figure 9(A) shows iCLEM imaging of a third ROI in the Mahogany Zone sample at the margin of a carbonate concretion or microbialite. Cathodoluminescence of diagenetic carbonate (shown by pinkish-red in Figs. 9A–C and E) is present within the interpreted concretion but is subdued in the adjacent organic-rich layer. Moreover, cathodoluminescence is present in the core of carbonate phases but absent in euhedral crystal overgrowths which terminate against sedimentary organic matter including both solid bitumen and amorphous material (Figs. 9E and F). Authigenic euhedral mineral terminations protrude into both the AOM (kerogen) and the solid bitumen. These textures may indicate diagenetic crystal growth before and during generation of early hydrocarbons in the Mahogany zone. Delivery of inorganic ions to the growing crystal face against the solid hydrocarbon (which we presume was mobile in the subsurface) implies that water was dissolved in the solid hydrocarbon phase (Lewan, 1997) or that an emulsion of water and bitumen was present during diagenesis.

Limitations

The optical microscopy portion of the iCLEM described herein is limited to low resolving power (~460 nm) due to the extra-long working distance 0.60 NA objective required to image fluorescence through the glass slide. In addition, presence of organic fluorescence generally is not observable above about 1.0–1.1% R₀ (Teichmüller & Durand, 1983), limiting the sample preparation and technique described to lower maturity geological samples. However, alternative sample preparation techniques may permit sequential incident white light oil immersion microscopy of uncovered samples followed by SEM in the SECOM. This would require a bridging-type transmission electron microscopy sample mount or a customized equivalent. Alternative iCLEM configurations (Nishiyama et al., 2010) may also permit incident white light optical microscopy under oil immersion with simultaneous inverted SEM through a silicon nitride film window. Sample preparation for either configuration would require thin foils produced via focused ion beam or microtome sectioning. Although more labour- and cost-intensive than the economical approach described herein which used standard thin sections, the alternative sample preparation and configuration approaches, if successful, would extend imaging and identification of organic matter to overmature conditions where fluorescence is extinguished but organic nanoporosity typically is well developed (Ko et al., 2016).

Summary

We have described an application of iCLEM to geological materials by sequentially imaging sedimentary organic matter in shale via fluorescence and SEM. The results show that iCLEM can be used to identify and characterize organic matter types based on their fluorescence response (most prevalent at lower thermal maturities of up to about 1.0–1.1% R₀) with simultaneous resolution of submicron features via SEM. Because higher magnifications are available via SEM, this technique also allows sequential imaging at scales ranging from hundreds of microns to nanometres, enabling identification of organic matter types via fluorescence microscopy at low resolution followed by high-resolution SEM evaluation of nanoscale structures such as organic porosity. Future work using iCLEM should investigate alternative instrument configurations and begin systematic evaluation of the thermal regime and organic matter types in which organic porosity develops, including...
shale samples of peak oil (VR₀ ~0.9 %) and higher maturities, and artificial maturation sequences created through hydrous pyrolysis experiments.

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