Correlative light and electron microscopy of poly(L-lactic acid) spherulites for automatic detection

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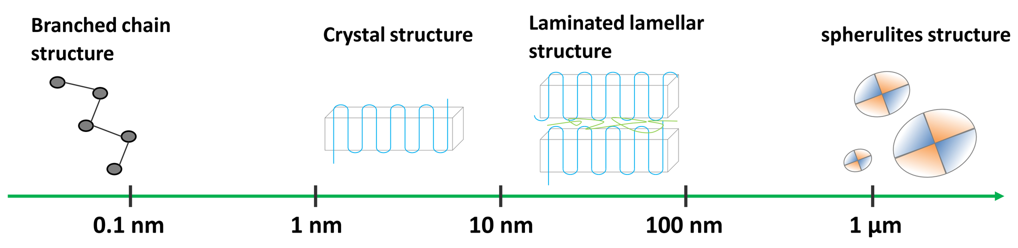
Abstract

Polarized optical microscopy (POM) and transmission electron microscopy (TEM) are widely used for imaging polymer spherulite structures. TEM provides nanometer resolution to image small spherulites of sub-micrometer in diameter, while POM is more suitable for observing large spherulites. However, high-resolution images with a large field of view are challenging to achieve due to the deformations of ultrathin sectioned samples used for TEM observations. In this study, we demonstrated that correlative light and electron microscopy (CLEM) combining POM and TEM could effectively characterize the spherulite structures in a wide range from nanometer to several hundred micrometers that neither TEM nor POM alone could cover. Furthermore, the deformations of the TEM ultrathin sections were corrected by referencing to the POM images at the same position of the sample, and large-area TEM images without deformations were successfully produced. The spherulite structures of poly(L-lactic acid) were successfully analyzed using CLEM and TEM in a wide range of spatial scales at the same field of view. The large-area TEM image (250 µm × 250 µm), consisting of 702 TEM images stitched together, was subjected to machine learning to extract the essential structural information of spherulites. Analysis using the convolutional neural network, a well-known algorithm You Only Look Once (YOLO), demonstrated that it was practical to accurately obtain the diameter distribution and the space-filling factor (relative crystallinity) of the spherulites. This study presents a new approach for acquiring high-resolution images with a large field of view and processing the images at a fast speed.

1. Introduction

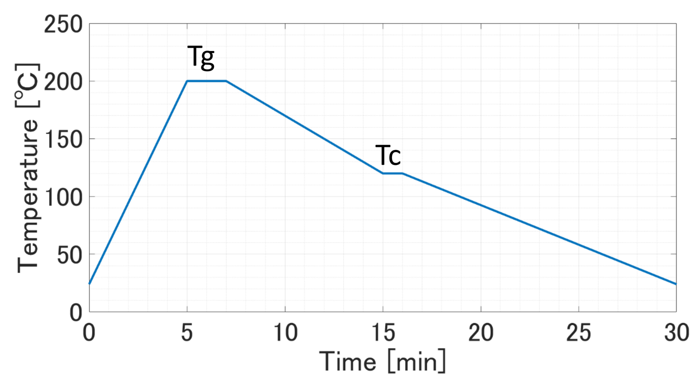
Crystalline polymers, such as polyethylene, are used in a wide range of applications because of their lightweight, low cost and excellent processability. The physical properties of crystalline polymers largely depend on their hierarchical solid-state structures with sizes spanning the nanometer to micrometer range. Therefore, understanding the hierarchical structure is essential for the structural design of crystalline polymeric materials. For this reason, it is desirable to have a method to analyze each level of the hierarchical structure at various scales. In the present study, we investigated poly(L-lactic acid) (PLLA), a crystalline polymer that has recently attracted attention as an environmentally friendly polymer derived from plants [23] using a CLEM system. We successfully overlaid the high-resolution TEM images onto the POM images using deformation correction. To match the field of view of the TEM and POM images, a large number of TEM images were stitched together to produce a high-resolution large-area TEM image. A convolutional neural network-based object detection system was applied to the large-area TEM image to detect each spherulite automatically. Finally, the diameter distribution and space-filling factor (relative crystallinity) of the spherulites within the desired region were calculated from the results.



2. Method

◆ Sample preparation

poly(L-lactic acid) (PLLA) -[-OCH(CH3)CO-]n-
- ☐ Thermal history is shown on the right
- ☐ Film thickness 13±2 µm

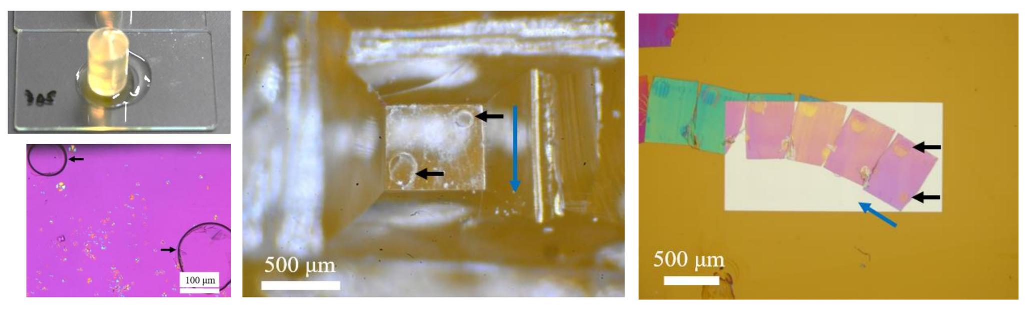


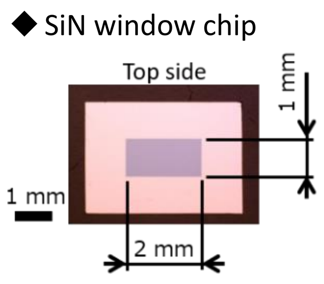
◆ POM observation

The spherulites of PLLA at Tc were observed
- ☐ POM Digital microscope (VHX-600)
  Temperature controlled stage (LTS350)
  Crossed Nicols optical system with a sensitive color plate

◆ Sample preparation for TEM

- ☐ Ultrathin sections Thickness of 200nm using an ultra microtome (Leica UC6)
- ☐ Sample support A chip with a SiN film (SiN window chip, JEOL Ltd.)



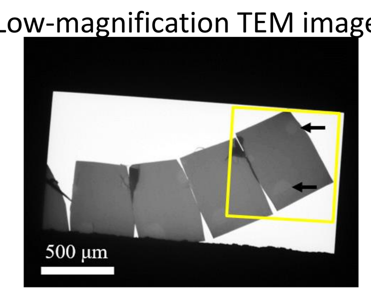


◆ Acquiring large-area TEM images

- ☐ TEM JEM-1400Flash (JEOL Ltd.)
- ☐ Montage Auto montage system "Limitless Panorama" (JEOL Ltd.)
  702 TEM images (27 x 26) were stitched together
- ☐ Imaging area Entire ultrathin section (470µm x 620µm)

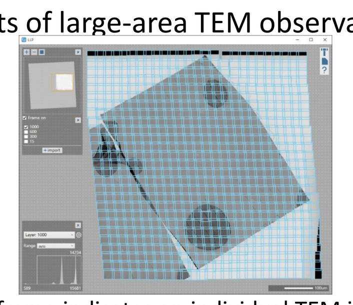
◆ JEM-1400Flash

◆ Low-magnification TEM image



The yellow frame indicates the imaging range of the large-area TEM image.

◆ Results of large-area TEM observation



Each blue frame indicates an individual TEM image, all of which were pieced together by Limitless Panorama to create this image.

◆ Overlay of POM and TEM images

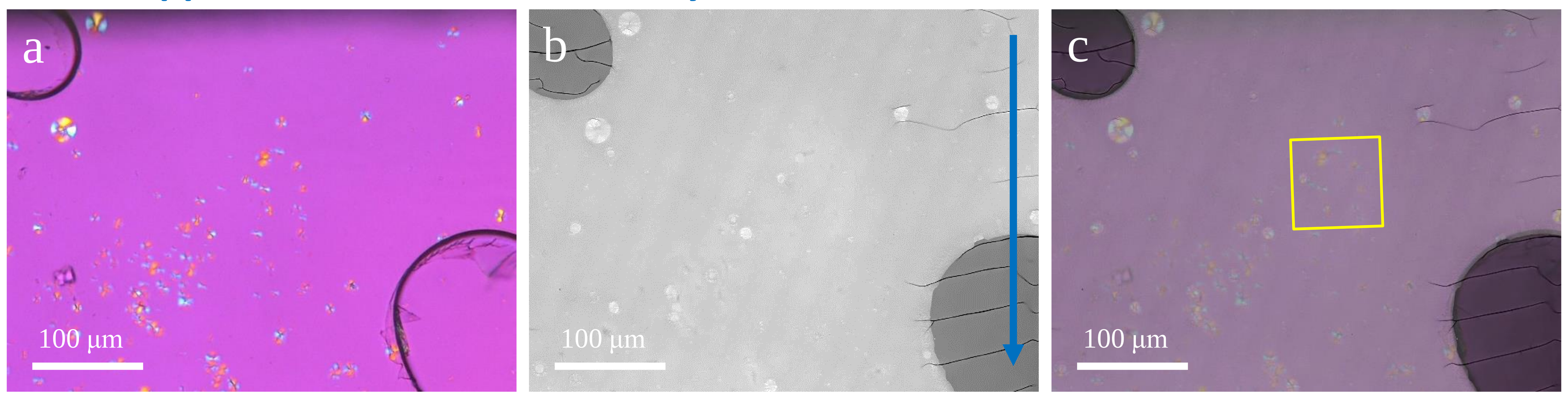
- ☐ Fiji and SightX viewer (JEOL Ltd.)

◆ Detection of spherulites and calculation of space-filling factor

- ☐ Detection of spherulites
  - ✓ Convolutional neural networks (CNN) : YOLOv5
  - ✓ Training :
    Among the dataset comprising 263 TEM images containing spherulites of PLLA
    - ➢ 213 images for the training set
    - ➢ 50 images for the test set
  - ✓ PC : Quadro P4000 with 8 GB in the Anaconda virtual environment
  - ✓ Time taken for training : about 11 hours
- ☐ Calculation of space-filling factor
  1. CNN generated by the training was used to detect the locations, widths and heights of the spherulites in the large-area TEM image.
  2. The width and height of each spherulite were used to calculate its area, assuming that the shape of the spherulite was an ellipse.
  3. The area calculated for each spherulite was summed up and divided by the area of the image to calculate the space-filling factor of the spherulites.

4. Result and discussion

◆ Application of CLEM to PLLA by POM and TEM

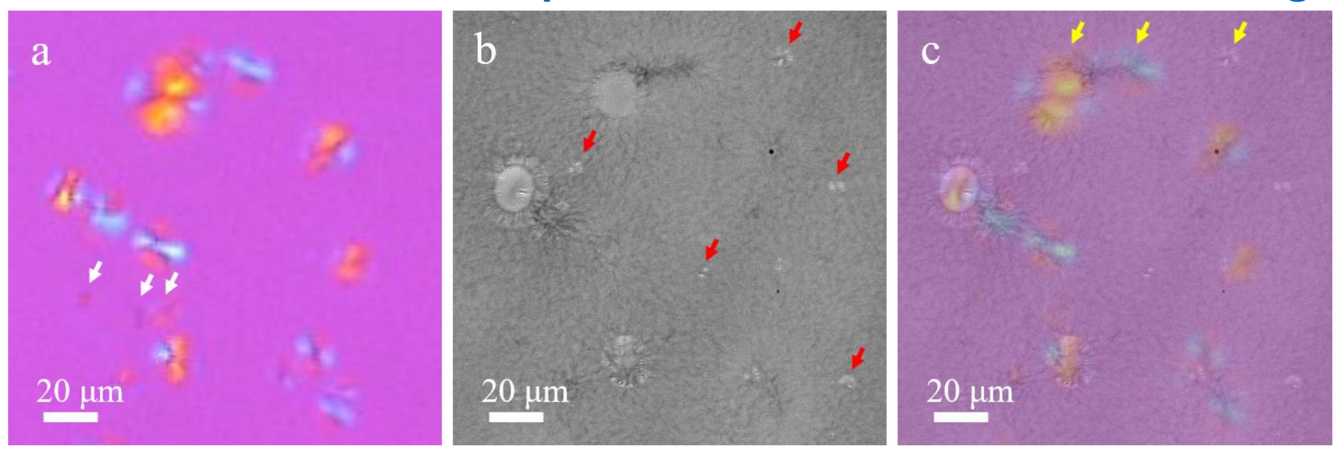


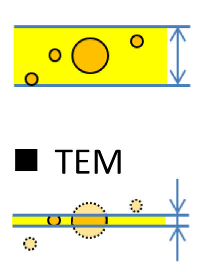
(a) POM image
- ✓ The molecular chains (c-axis) were oriented perpendicularly to the radius of the spherulites in the interior of the lamellar crystal growing radially from the center.
- ✓ Yellow color in the first and third quadrants and blue color in the second and fourth quadrants indicated the negative birefringence.

(b) Large-area TEM image of the same field of view as the POM image
(deformations caused during ultrathin section preparation have been corrected)
- ✓ The microtomed sample for TEM was stretched about 1.16 times in the direction perpendicular to the cutting direction due to stress during the preparation of ultrathin sections.

(c) Merged image of the POM and TEM images.

◆ Difference between the spherulites in the POM and TEM images

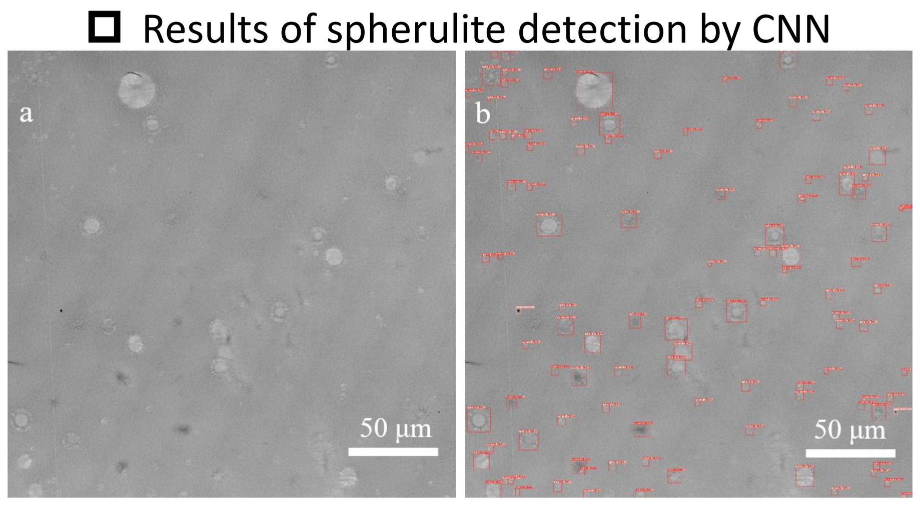




(a) POM image, (b) Large-area TEM image, (c) Merged image of the POM and TEM images.
- ✓ The spherulites marked with red arrows are visible only in TEM images
  - ➢ The sizes of the spherulites were smaller than the POM resolution (~250 nm)
- ✓ The spherulites marked with white arrows are visible only in POM
  - ➢ They were positioned either above or below the TEM section along the film thickness direction.

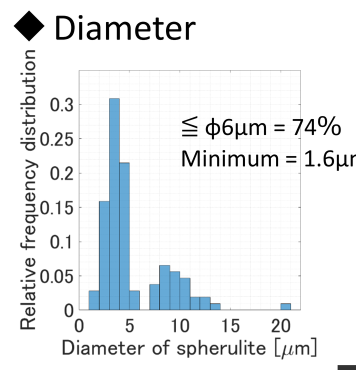
◆ Detection of spherulites and calculation of space-filling factor by CNN

☐ Results of spherulite detection by CNN

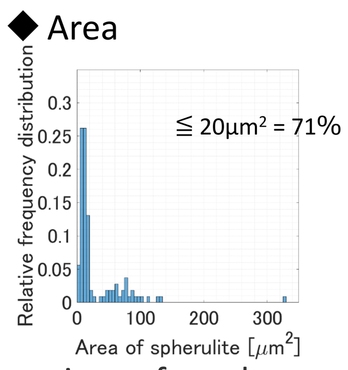


☐ Relative frequency distribution of spherulites

◆ Diameter



◆ Area



☐ Comparison of result

Image	Space-filling factor	Detection method	Processing time
TEM	5.2 %	YOLOv5	**3 sec**
	6.3 %	Manual	30 min
POM	8.3 %		

- ✓ Detection factor (vs visual observation) : **81%**
  Number of detections : 107, Number density : 0.0017 /µm²
- ✓ Space-filling factor : **5.2%** (relative value)
  - Crystalline and amorphous regions coexist inside the spherulites
  - Space-filling factor including the undetected spherulites : **6.3%**
    5.2% / 0.81 = 6.4% → Approximately equal values using the detection factor
- ➢ This method can be used to estimate the space-filling factor of spherulites (relative crystallinity) within a desired region.

Summary

- ◆ Using POM and TEM, the spherulitic structure of PLLA was observed in the same field of view.
- ◆ The combination of both microscopes in the CLEM system made it possible to obtain large-area TEM images without the concern of sample deformation and enabled us to observe tiny spherulites that POM was not able to detect.
- ◆ We proposed a method to detect spherulites from the acquired large-area TEM image using YOLO, a well-known object detection system using convolutional neural networks, and obtained the number density and space-filling factor (relative crystallinity) of spherulites within the desired region.
- ◆ The present method would be more effective than the conventional methods in analyzing the heterogeneously distributed spherulites in materials.