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Moon, Robert; Johnston, Linda; Land-Hensdal, Cecilia; Batchelor, Warren

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Perspectives on cellulose nanofibril size measurement using scanning electron microscopy

Robert Moon · Linda Johnston ·
Cecilia Land-Hensdal · Warren Batchelor

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Abstract Cellulose nanofibril suspensions present a broad range of particle morphology and dimensions spanning from millimeters to nanometers. As a result, direct imaging and indirect scattering approaches are used to quantify the morphology and dimensions across different length scales. There is a notable gap in detailed size measurement of cellulose nanofibrils produced from the mechanical refining of woody plants, which makes the required characterization for production control, grade specification, product specifications, and compliance with safety/regulatory requirements difficult. The cellulose nanofibril particles produced by mechanical treatment have

a morphology that is dominated by a hierarchical branched fibrillar structure, in which a thicker central fibril branches off into thinner fibrillar elements, which may also undergo further branching into even finer fibrillar elements. The large differences in dimensional scales between fibril length (micrometers) to that of fibril width (nanometers) within a given nanofibrillated cellulose object makes it difficult to measure, as well as to identify the relevant features to measure and report. This paper provides a perspective on scanning electron microscopy (SEM) as a method to partially address this issue. SEM imaging offers a reasonable balance between ease of use, measurement time, image quality, and versatility in magnification to enable size characterization and assessment of features across the variable length scales of the hierarchical branching. This paper also provides a summary of useful SEM techniques for CNF size measurements and practical guidelines for sample preparation, fibril diameter measurement, and methods to account for hierarchical branching. Finally, a comprehensive set of guidelines for measurement reporting is given, together with a discussion of future directions.

Robert Moon, Linda Johnston, Cecilia Land-Hensdal and Warren Batchelor have contributed equally.

R. Moon
The Forest Products Laboratory, USDA Forest Service,
Madison, WI 53726, USA
e-mail: Robert.j.moon@usda.gov

L. Johnston
Metrology Research Centre, National Research Council
Canada, Ottawa, ON K1A 0R6, Canada
e-mail: Linda.johnston@nrc-cnrc.gc.ca

C. Land-Hensdal
Stora Enso Karlstad Research Centre, Karlstad, Sweden
e-mail: cecilia.land-hensdal@storaenso.com

W. Batchelor (✉)
Department of Chemical and Biological Engineering,
BioPRIA, Monash University, Clayton 3800, Australia
e-mail: warren.batchelor@monash.edu

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Introduction

Cellulose nanomaterials, defined as cellulose-based materials with at least one dimension less than 100 nm, have undergone a forty-year evolution from initial reports of their production and characterization to a diverse range of commercial applications (Dufresne 2019; Foster et al. 2018; Moon et al. 2011; Nechyporchuk et al. 2016). As commercialization expands, the need for precise measurement techniques becomes paramount (Beck et al. 2019). Commercial producers require methodologies to ensure consistent quality and understand material characteristics crucial for performance. In research laboratories, challenges persist in material identification and classification, particularly in regulatory contexts such as human contact, food, or medical applications. The complexity increases when considering the diversity of cellulose nanomaterials, ranging from nanospheres to low-aspect ratio nanocrystals, oxidized nanofibers, and branched mechanically separated materials, each presenting unique measurement challenges.

There are three general classes of cellulose nanomaterials produced from woody plants that differ in particle morphology and size distributions: cellulose nanocrystals (CNCs), individualized cellulose nanofibrils (iCNFs) and cellulose nanofibrils (CNFs). Approaches for the measurement of CNC and iCNF particle length, width and height have been developed through the International Organization for Standardization (ISO) (ISO 2021a, 2021c). In contrast, there are no technical specifications for general CNF particle size measurement because its complicated branched fibrillar structure makes it difficult to identify the relevant features to measure and report. For example, typical CNFs have a wide distribution of fibril diameters, including their branches, and it is unclear in many cases whether they are fully separated or have become entangled during the process of preparing the sample for imaging. While the diameters of the individual components that make up a CNF object can be directly measured using imaging techniques, lengths cannot, as it is nearly impossible to identify the start and end of individual fibril elements. Note that the recent ISO standard for electron microscopy (ISO 2020) include data analysis methods for nanosized objects (both qualitative and quantitative); however, they are difficult to apply to CNFs due to their more challenging morphology.

The authors of this report are part of an ISO task group working on standards for CNF characterization. From a survey of producers, researchers and end-users, the following areas were previously identified as the most urgent (tier 1) for standards development: dry matter content, viscosity and rheology, particle size and particle size distribution, pH, dispersibility in aqueous media, specific surface area and degree of agglomeration (Beck et al. 2019).

Subsequently further work was conducted on standards for dimension measurements. The process followed and the outcomes are discussed in detail in (Moon et al. 2023). Briefly, a survey was set up, with results collected separately for companies and individual researchers. This was followed by a two-day workshop in 2021 to consider the survey findings and to conduct a strength-weakness-opportunities-threats (SWOT) analysis of the different CNF particle morphology measurands and their measurement problems. Consensus emerged across stakeholders (industry and academia), transcending geographical and application-specific boundaries, of the critical importance of measuring CNF fibril length and diameter for production control, grade specification, product specifications, and compliance with safety/regulatory requirements. The inability to adequately characterize the nanosized hierarchical branched structures within CNFs negatively impacts the commercialization of CNFs as these features strongly influence CNF physical properties (degree of entanglement), chemical properties (surface area available for modification), optical properties (transparency in suspension and film forms), and their interaction with the environment. Thus, improved methods and guidelines are needed for CNF particle size determination and the assessment of the nanosized hierarchical branched structures.

A notable gap in CNF particle size measurement that was identified was the inability to concurrently measure across large differences in length scales (micrometers for fibril length and nanometers for fibril width) (Moon et al. 2023). Scanning electron microscopy (SEM) was one method that was considered to partially address this issue as it offers versatility in imaging, enabling size characterization and assessment of additional features such as branching, where bulk measurement techniques falter. SEM imaging provides a reasonable balance between measurement time and image quality, and thus has

relevance and feasibility for applicability for both industry and academia. As a result of these factors enhancing SEM methodologies for CNF size measurement emerged as a primary objective from the workshop (Moon et al. 2023).

Several fundamental and practical issues were identified for dry-imaging techniques like SEM that, if addressed, would greatly improve CNF particle size measurement and facilitate subsequent development of standards. (1) how can samples be reproducibly prepared to minimize agglomeration of hierarchical branched CNF structures during drying, (2) can guidelines for fibril diameter determination be developed that can fully characterize and distinguish the hierarchical branched CNF structures, (3) how can CNFs be classified to encompass measurements at multiple size ranges from micro- to nanoscale to fully characterize and distinguish CNF samples, and (4) can adequate automated image analysis programs that can identify and measure complicated hierarchical branched CNF structures be developed? There is a relevant standard for SEM measurement of nanomaterials (ISO 2021b), which is a good starting point for CNF particle size standards development via SEM; however, it deals with nanomaterials in general and is thus difficult to apply to CNFs which have a more challenging particle morphology and are difficult to image.

This paper aims to address these issues by providing a review of SEM techniques for CNF size measurements, and offering best practices guidelines for sample preparation, fibril diameter measurement, and accounting methods for hierarchical branching. By

addressing methodological nuances and highlighting best practices, this paper will contribute to the advancement of CNF size analysis.

Background

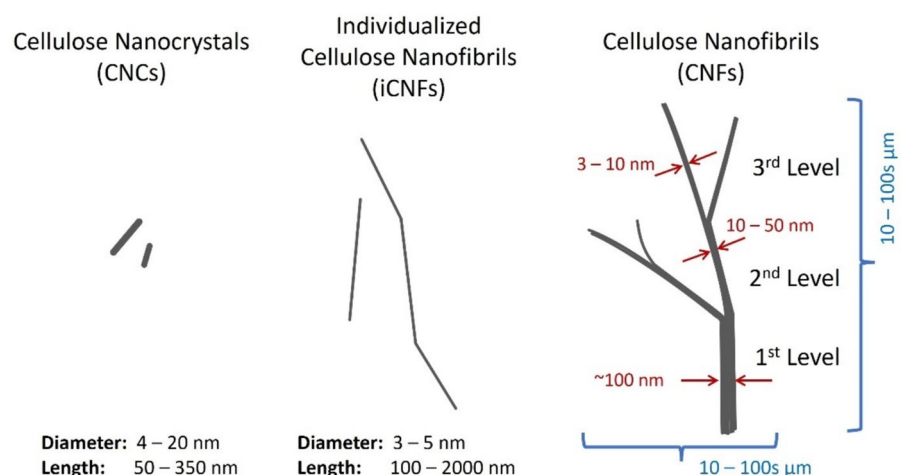
Cellulose nanomaterial types

(ISO 2023) divides cellulose nanomaterials, which can also be called nanocellulose, into three general classes of cellulose nanocrystals (CNCs), individualized cellulose nanofibrils (iCNFs) and cellulose nanofibrils in general (CNFs). A schematic diagram of the general differences between the classes is given in Fig. 1, while Fig. 2 summarizes the general features of each class of material, their size ranges and the most commonly used techniques to measure size. The size ranges are then summarized graphically in Fig. 3.

CNCs are low aspect ratio, small diameter rod-like (spindle) particles typically prepared by acid hydrolysis. While the small diameter limits measurement principally to either AFM or TEM, the lower aspect ratio simplifies the analysis, as both length and width dimensions can be measured on the same image scale. Methods for CNC particle sizing using either AFM or TEM are provided in (ISO 2021c) and have been validated in an international interlaboratory comparison (Bushell et al. 2021; Meija et al. 2020).

iCNFs are typically prepared using oxidation and mechanical treatment. The resulting fibrils have diameters close to that of an elementary fibril (3–5 nm),

Fig. 1 Schematic of particle morphologies for CNCs, iCNFs, and CNFs. The CNF object illustrated here has a three-level hierarchical branched framework. We define each branch as a fibril element



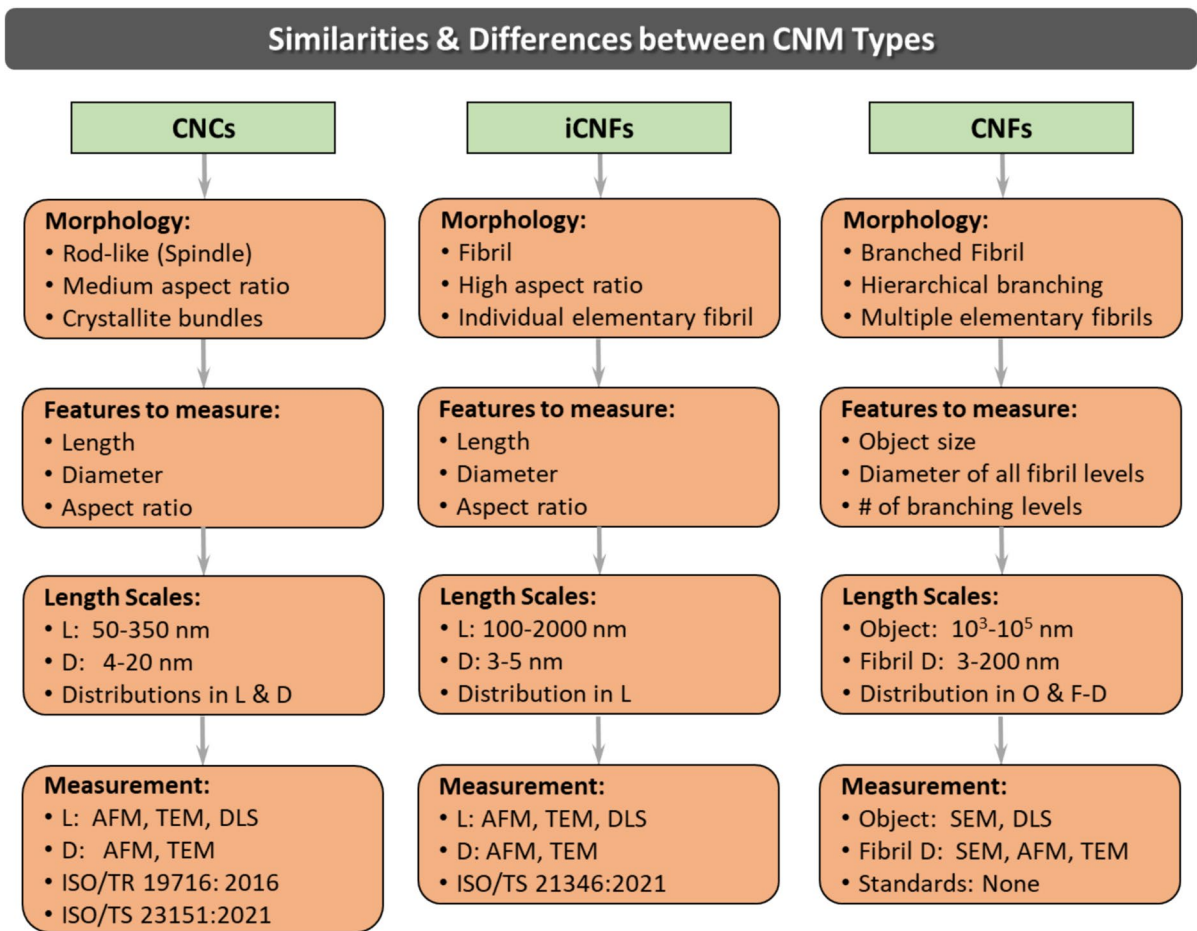


Fig. 2 The three classes of woody plant-based cellulose nano-materials (CNCs, iCNFs, and CNFs), listing the similarity and differences across four parameters relevant to characterizing their particle morphologies and feature sizes (Length (L), Diameter (D)). Note that the cross-section is not always spherical, and the term diameter is used to indicate the width that can

be measured in TEM/SEM or the height that can be measured in AFM. A CNF object is defined as a hierarchical branched structure as illustrated in Fig. 1. AFM is atomic force microscopy, DLS is dynamic light scattering, TEM is transmission electron microscopy

with frequent kinks and a high aspect ratio, which increases the complexity of analysis by increasing the probability that length and width dimensions need to be measured on different image scales, and the likelihood of fibril-fibril entanglement. Characterization methods for iCNFs are summarized in (ISO 2021a), which includes measurements of morphology and size by AFM and TEM that have the required resolution to measure a fibril diameter of 3–5 nm.

CNFs are prepared using mechanical treatment. The resulting objects are typically composed of bundles of elementary fibrils that can contain branches, a significant fraction of which are in the

nanoscale. Though CNF dimensions are typically reported to be 3–100 nm in cross-section and typically up to 100 μ m in length, realistically there is some ambiguity in determining a typical CNF object size, as CNFs can frequently form entanglements between particles or network-like structures when the distance between fibrils is sufficiently close. Additionally, the complex hierarchical branched framework shown schematically in Fig. 1 is a significant complication for size measurements, as such an object cannot be characterized by a single diameter or length.

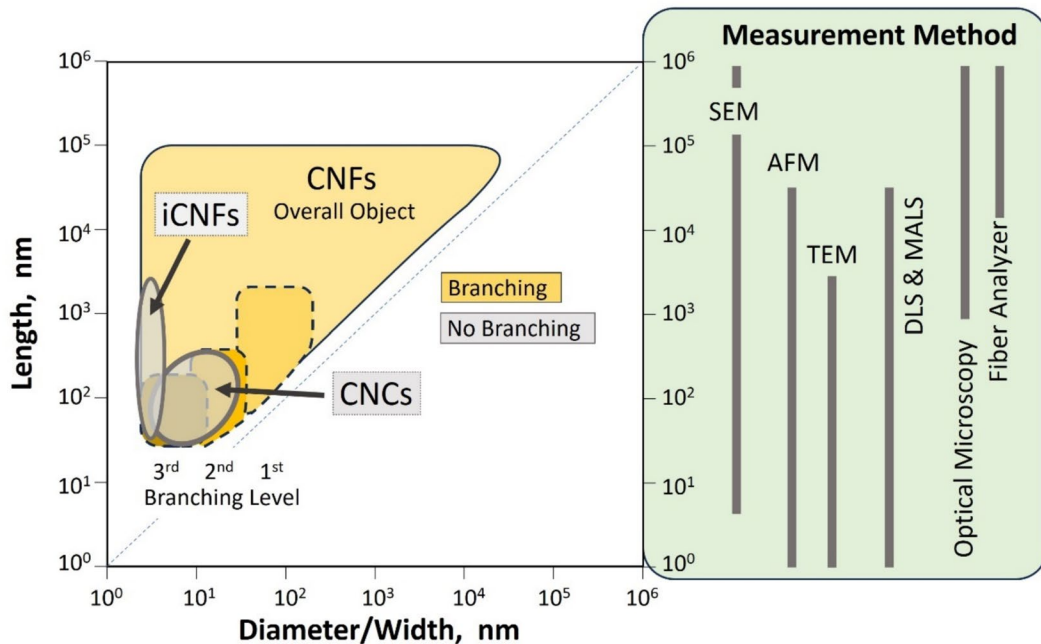


Fig. 3 Schematic representation of the approximate dimensions of CNCs, iCNFs, and CNFs. For the CNFs, additional dashed shapes represent a three-level hierarchical branched framework in Fig. 1, showing the corresponding length and

width for each branch level. To the right of the plot the approximate range of feature sizes that can be measured for several techniques is summarized. MALS is multiangle light scattering

Challenges in CNF morphology measurement

The mechanical fibrilization processes used to make CNFs result in suspensions that contain particles with various morphologies, having a range of sizes spanning millimeters to nanometers (Kangas et al. 2014; Nechyporchuk et al. 2016; Wang et al. 2012). The larger millimeter sized objects (e.g., pulp fibers and fiber fragments) are often removed by fractionation. The resulting CNF suspension contains micrometer to nanometer sized objects, such as microfibril bundles, hierarchical branched tree-like structured fibrils and fibrils with few branches. To characterize particle size across this wide size scale requires multiple measurement approaches and techniques (e.g. optical microscopy, fiber analyzer, DLS, SEM, AFM, and TEM) (Kangas et al. 2014). The focus of this article is the measurement of the morphology of CNFs that have a typical hierarchical branched tree-like structure of either individual fibrils or fibrils attached to larger microfibril bundles.

The fibrillated morphology of CNF materials (Fig. 1) is considerably more complicated than

conventional inorganic nanomaterials, or even CNCs and iCNFs. The challenges of CNF morphology and size measurement stem from the branching and interconnected structures of the cellulose fibril elements, making it difficult to identify the relevant features to measure and report. There are various factors to consider. For example, it is unclear in many cases whether CNF fibril elements are fully separated or have become entangled during the process of preparing the sample for imaging. Should one measure an average width of each branch or measure the width at a specific point, either the maximum or minimum width or possibly the midpoint of the branch if that can be determined? How important is it to measure all branches and can this reasonably be done on images obtained with a single magnification or will multiple magnifications be needed? While diameters of fibril elements can be directly measured, lengths often cannot, as it is difficult to identify the start and end of individual fibril elements. Should the number of branching points, and fibril intersection points be counted? How can one assess the nanosized fraction of a given CNF object? How many particles must be

analyzed to have a reproducible and representative measurement for a specific material?

The above factors are all important for measuring CNF size for quality control of material production and application development. These factors are also important for product safety and regulatory purposes for which there is currently a lack of understanding of the information on CNF size and morphology that will be needed for product approval. To classify whether the material should be considered as a nanomaterial or not, representative measurements of the cross section of the object are needed since it is this dimension that will determine whether the material meets the criterion for classification as a nanomaterial.

SEM imaging of CNFs for size measurement

General overview

To overcome many of the challenges in measuring CNF morphology and the branched fibril structure, it is relevant to consider the capabilities of SEM as well as comparing it to AFM and TEM imaging.

A SEM scans a sample surface with a focused electron beam in a vacuum. The information detected is used to present an enlarged image of the sample surface. The factors that determine the ability to obtain high resolution SEM images to capture the nanoscale complexity of CNF objects include a small electron source diameter, a high electron emission (brightness) and a small energy width.

The type of electron gun defines the type of SEM (conventional tungsten or lanthanum based, Field Emission etc.). The Field Emission Gun SEM (FE-SEM or FEG-SEM) systems have the highest resolution and can in many cases be appropriate for size analysis of CNFs since they allow visualization of cellulose fibril elements with diameters as small as ~10 nm. The actual resolution is also sample dependent. Some SEM options available, that will not be discussed further as they have not been reported in the literature for CNF particle size measurement, include Variable Pressure SEM/Environmental SEM (ESEM) and Cryo SEM.

The choice of detector depends on the analysis that is to be performed. A secondary electron (SE) detector captures low energy electrons that escape from

depths up to a few nm and is mostly used for imaging the surface, while a backscatter detector captures higher energy electrons from a larger region and is sensitive to composition of the material. Other detector types for other purposes are also available.

Secondary electron detectors can be positioned to the side (lateral SE detector) or positioned in front of or within the objective lens (in-lens detectors) (Zhang et al. 2016). The most common geometry is the lateral type, with the ETD (Everhart–Thornley Detector) being the most frequently used (Zhang et al. 2016). In-lens detectors can operate at shorter working distances and generally result in a stronger secondary electron signal for a given acceleration voltage and provide higher resolution imaging. Figure 4 gives an example of imaging CNF with lateral, and with in-lens SE detectors. Lateral SE detectors give topographic information of CNFs, while in-lens SE detectors give more contrast between the CNF material and the substrate, which can facilitate automated measurement of fibril dimensions. Backscatter detectors can give images with even higher contrast based on the local atomic number composition of the sample. A conductive surface is generally needed for imaging with SEM. With non-conductive samples, such as CNF, a thin conductive coating is usually applied. The conductive coating prevents charging of the sample, protects it from damage and improves the information content of the image.

The wider range of available magnifications of SEM imaging makes it possible to measure a broader range of widths and lengths of the hierarchical fibril branching within a given CNF object as compared to AFM or TEM (Fig. 3). This gives the capability to measure both length and widths on different image scales, providing improved linkage of fibril length to width measurements of fibril elements. However, the minimum size measurable of cellulose fibrils by SEM (~10 nm) is larger than that achievable with AFM or TEM (~2 nm), and may prevent measurement of the diameter of the smallest fibrils. Despite this, SEM is generally significantly faster than AFM in image acquisition, while compared to TEM it is less expensive, more readily accessible, easier to use, and has easier sample preparation. It should be noted that despite these relative advantages, high resolution SEM is often not available in commercial production environments due to cost, although it is generally

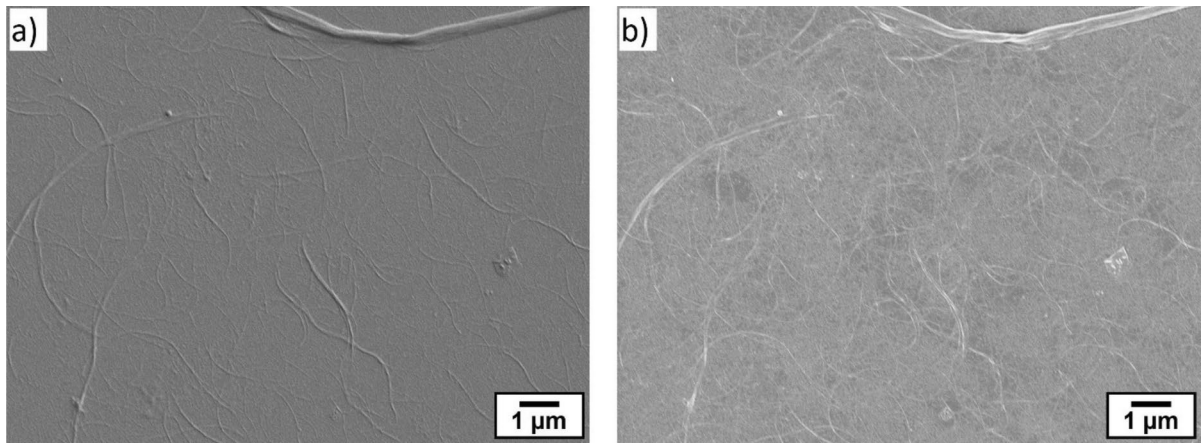


Fig. 4 Scanning electron microscopy images of CNF acquired with FE-SEM (Zeiss Sigma VP, 1.5 kV, working distance of 6 mm) using **a** lateral SE detector, and **b** in-lens SE detector. The in-lens SE detector shows higher contrast between

the CNF and the substrate. The SEM sample preparation: Poly(ethylene imine)-treated mica discs were dip coated with CNF, and then coated with a 4 nm gold layer. Images adapted from (Mattos et al. 2019)

available in research laboratories. In a few cases TEM has been used to measure the smallest fibril widths for highly processed CNF samples (Pyrgiotakis et al. 2018; Rol et al. 2019; Wang et al. 2012).

Typically, SEM of CNFs has been used to provide a qualitative assessment of the overall morphology and dimensions of the sample. Fibril size measurements based on analysis of a sufficient number of particles to define the particle width distribution are reasonably rare in much of the published literature. This is the case even for examples where good quality images with reasonable levels of sample dispersion have been obtained. Furthermore, CNFs have been produced by a range of different methods from many different sources of cellulose biomass, making it difficult to compare the utility of sample preparation methods across different studies. These issues are compounded by a frequent lack of details on sample preparation, imaging and data analysis which makes it difficult to reproduce experiments and compare to other studies.

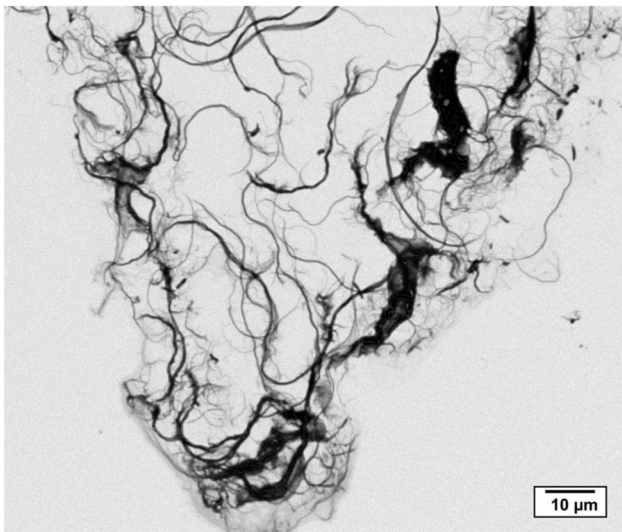
In the following sections we discuss the use of SEM for morphology measurements of CNF, based both on the literature as well as our practical experience working in this area. The different steps in the workflow that need to be considered are shown in Fig. 5.

CNF heterogeneity and sample pre-analysis/fractionation

It is critical to consider CNF suspensions as a mixture of different sized particles, and because of this heterogeneity we believe that it is essential that, prior to any SEM imaging and subsequent image analysis of nanosized particles, the sample undergoes basic analysis to determine the volume and size distribution of larger sized particles (e.g., pulp fiber fragments and microfibrillated bundles) in the sample. This information is relevant when assessing the fraction of nanosized objects within the CNF suspension. We acknowledge that researchers have not always conducted this preliminary analysis. In many cases it may be necessary or desirable to remove larger particles (Fig. 6a, b) prior to a more detailed size analysis using SEM (Fig. 6c, d).

One widely available measurement technique is the use of a fiber analyzer such as MORFI or Fibrelab to identify the pulp fibers, fiber fragments, and larger fines in the sample. Fiber analyzers are designed around detecting papermaking fibers with lengths typically 0.2 to 5 mm. Such analyzers use optical microscopy and are rapid and require little sample preparation. Automated fiber analyzers generally report fiber properties at two or more length classes. The objects shorter than the fiber limit of either

Flow Chart for SEM Particle Size Measurement of CNFs:



1 Pre-Analysis

Fractionation (micro-fines screening, centrifugation)
Assess larger sized particles (Fiber analyzer)

2 Sample Preparation

Dilution, dispersion on substrate, substrate coatings

3 SEM Methods

Conventional SEM, Negative Contrast SEM

4 Imaging Requirements

Multiple magnification, number of images
Where to take images in sample

5 Image Analysis

Features to measure, analysis approach, statistical analysis

6 Reporting

CNF preparation, fractionation, pre-analysis results
SEM sample preparation, imaging parameters
Analysis details, statistical analysis approach

Fig. 5 Negative contrast SEM image of a CNF object (UMaine -90% fines CNF), showing a complex branching structure. Flow chart of steps necessary for CNF size measurement and reporting using SEM techniques. Image was adapted from (Ringania 2023)

0.1 mm (Tappi/ANSI 2023) or 0.2 mm (ISO 2014a, 2014b) are generally considered as fines, with the lower limit depending on the resolution of the imaging system within the instrument. Detailed characterization is generally not available for the fines, but sometimes they can be divided into two sub classes. As an example, Carter (Carter et al. 2021) produced CNFs through low consistency mechanical refining, but even after 90 min of refining, the fiber analyzer showed that although nearly half of measured fibers were in the lowest fiber length measurable, still 0.4% of fibers were 1 mm long or more. The limitation of this type of measurement is that while large fibers and fragments can be detected, the method is unable to show the presence of nanofibers. It might be possible to partially bridge this gap by instruments such as Diamscope and Qicpic that have been designed for other applications than wood fibre suspensions, but the lowest diameter that can be measured is still 200 nm.

If significant numbers of large fibers and fiber fragments are identified, it is recommended to separate them from the sample before SEM imaging to improve the characterization of the nanosized particles of CNF suspensions. There are several possible

approaches, such as centrifugation (Larsson et al. 2019; Zhai et al. 2020), flow fractionation (Kangas et al. 2014) and filtration (Larsson et al. 2019). Zhai et al. (Zhai et al. 2020) performed centrifugation on the CNF samples, performing first a low speed centrifugation to remove the large fiber fragments and then a high speed extended run to remove the very fine CNF. By selecting the time and rotational speed, fibers and fiber fragments can be removed, leaving the finer material in suspension. Gravimetric analysis can then be used to identify the weight fraction of finer and coarser material. Subsequent SEM analysis will be easier and more reliable if the larger fragments are removed. It is notable in this analysis that the middle CNF fraction was only 18% by weight.

Another approach for removing large fibers is to screen the sample with a 200-mesh screen. The mass fraction passing through the screen is considered fines for conventional wood fiber samples. After screening through a 200-mesh screen, subsequent finer screens such as a 400-mesh screen can be used. This process will exclude all larger material and somewhat reduce the heterogeneity of the sample.

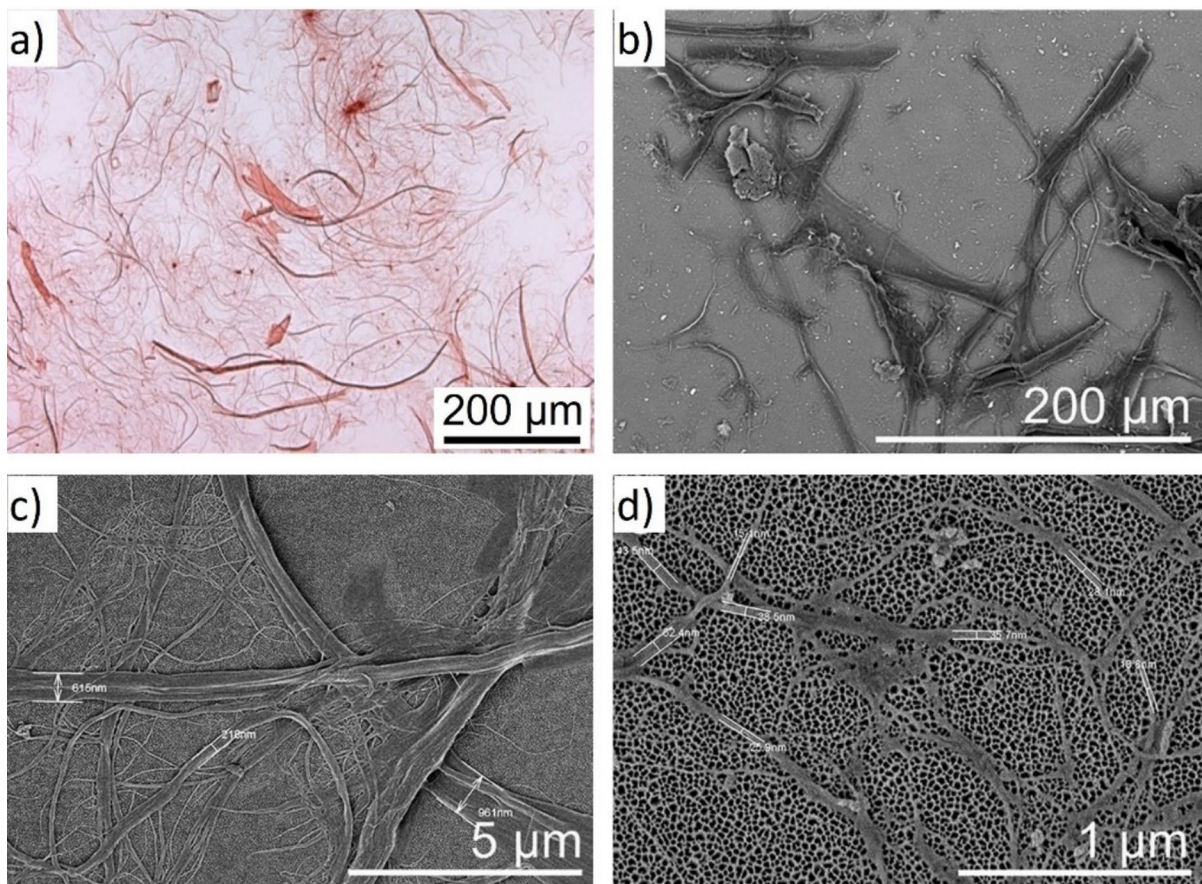


Fig. 6 The heterogeneity of particle sizes in CNF suspensions: **a** Optical image of a commercial CNF (Celish KY-100G, Daicel) showing a coarse size range of fiber-like particles. Adapted from (Kangas et al. 2014). **b–d** SEM images of a commercial CNF (MFC, Borregaard AS), demonstrating that

conventional SEM can image both the coarse **b**, and the finer size range **c** and **d** of the hierarchical branched fibril structures of CNFs, (Larsson et al. 2019). The small pore structures in **c** and **d** are from the membrane used in the sample preparation

SEM sample preparation

Proper sample preparation is important to ensure that features of interest are isolated and clearly visible, facilitating reliable size measurements using microscopic methods. The ideal is a well-dispersed sample that is representative of the bulk material and for which agglomeration and particle–particle contact are minimized while achieving a uniform particle distribution to facilitate analysis of a sufficient number of particles with a minimum number of images collected (ISO 2021b). One should also select a substrate that enhances the contrast between particles and background.

Routine preparation of suitable samples can be challenging for most cellulose nanomaterials as they are high aspect ratio materials with a strong tendency to agglomerate and aggregate and have poor contrast compared to, for example, most inorganic nanoparticles. However, published results have demonstrated that acceptable results and reliable particle size distributions can be achieved for both CNCs and iCNCs by careful attention to methods. For example, for CNCs the use of spin coating on a poly-L-lysine (PLL) coated slide has shown to be useful to minimize support-induced agglomeration and maximize the number of CNCs per image (Jakubek et al. 2018). Similarly, iCNCs can be

well-dispersed for analysis by either AFM or TEM (Usov et al. 2015).

Taking the above limitations into account we focus here on a number of recent studies in which SEM has been used to obtain morphological and dimensional information on CNFs and, in some cases, correlating the results with AFM and/or optical microscopy. Many of these examples attempt to maximize dispersion of the sample on the substrate by starting with very dilute CNF suspensions (eg, 0.001 wt % in water) that have often been pre-treated by vortexing or sonication to minimize aggregation (Ang et al. 2020; Bitounis et al. 2019; Pyrgiotakis et al. 2018; Ringania et al. 2022). When pre-treating with sonication, one should validate that the sonication energy used does not cause unintended consequences such as damage or altered CNF particle morphology (Bitounis et al. 2019; Zhang et al. 2024). This can be accomplished by monitoring size/morphology as a function of sonication energy. Another commonly used strategy is to deposit the CNF sample on a PLL or polyethyleneimine coated substrate such as mica or silicon in order to minimize particle–particle interactions (Pyrgiotakis et al. 2018); note that the attractive force between the coating and CNFs has been hypothesized to cause a flattening of fibrillar elements, subsequently increasing their measured widths (Mattos et al. 2019).

Preparation of samples by freezing and subsequent lyophilization has also shown promise as a method to minimize any possible redistribution and agglomeration during sample drying on the substrate and to maintain as much as possible the sample organization in the original suspension (Kangas et al. 2014).

Examples highlighting freeze drying and evaporation casting are found in Fig. 7. Another approach has been to use a nanoporous membrane to filter the suspension under vacuum (Fig. 6c, d). This approach can be effective in avoiding the aggregation of CNFs produced by capillary forces that can occur when individual drops of suspension are evaporated (Larsson et al. 2019).

Based on the above examples, it appears that depositing a dilute CNF suspension (0.001 wt%) by drop casting on a substrate coated with a positively charged polymer (rather than immersing the substrate in CNF suspension) and then rinsing the sample to remove loosely adhered material is a good starting point. For conventional SEM the best results have been obtained by coating the sample with a thin metal layer or adding a stain for improved contrast (Wang et al. 2012). When preparing samples for negative contrast SEM (discussed in the next section), good results have been obtained by depositing the sample on a gold or metal-coated substrate indicating that using a dilute CNF suspension is also adequate in this case. It is highly recommended to try several concentrations of suspension to find the optimal particle density/dispersion.

SEM methods

The most common practice in the literature for SEM measurement of CNF dimensions has been to use a secondary electron detector, conventional sample configurations and metal coated samples. Example SEM images of CNFs are shown in Fig. 7 for several different samples and sample preparation methods.

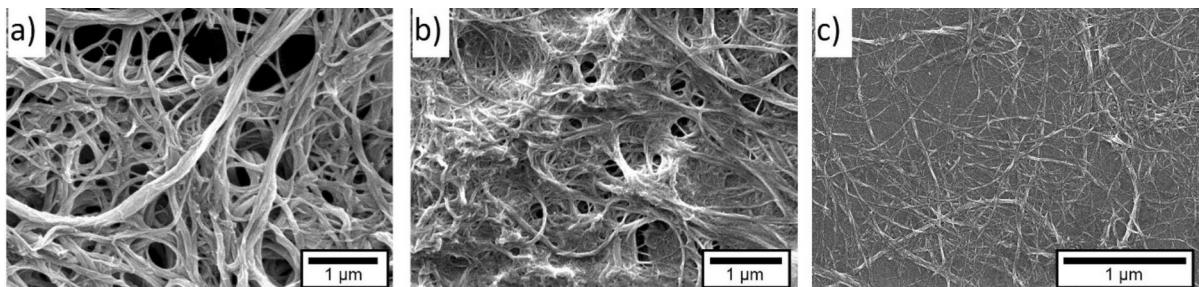


Fig. 7 Typical images from the following conventional SEM techniques: **a** Daicel (Celish KY-100G), FE-SEM, freeze dried sample preparation. Adapted from (Kangas et al. 2014) **b** VTT, FE-SEM, freeze dried sample preparation. Adapted from (Kan-

gas et al. 2014), and **c** FE-SEM, evaporation cast sample preparation. Adapted from (Ang et al. 2020) and reproduced with permission from Springer Nature

Imaging with a higher concentration of CNFs on substrates, like in Fig. 7 are adequate for measuring fibril width. Independent of the type of experimental setup, it is important to ensure that the microscope has been calibrated.

Negative-Contrast Scanning electron microscopy (NegC-SEM) has recently been shown to produce significantly higher contrast images of cellulose nanomaterials (Fig. 8). The conventional SEM sample configuration for non-conducting materials, such as CNF, is to deposit a conductive metal (e.g., gold, platinum, etc.) layer that coats the non-conducting material to minimize charging effects and which results in a more uniform secondary electron intensity across the imaging area (Fig. 8a). For NegC-SEM, the material to be imaged is deposited directly on an atomically smooth and highly conductive substrate. Both backscattering (Ringania et al. 2023) and lateral and in-lens secondary electron (Mattos-2019) have been used. The intensity of secondary electrons generated from CNFs is much lower than that of the substrate, thus a high contrast image is generated between the conductive substrate and the non-conductive CNF (Fig. 8b). This high contrast facilitates image analysis of CNF morphology, and fibril length and widths across various length scales. For improved image resolution, it is possible to use FE-SEM at low accelerating voltages (e.g. 1.5 kV) with in-lens secondary electron detector at shorter working distances (e.g. 6 mm) (Beaumont et al. 2021; Mattos et al. 2019). Various types of conductive substrates can be used. Ringania et al. (Ringania et al. 2022) used a silicon wafer (resistivity: 1–30 Ohm-cm, P-type with no SiO₂ top coating,

460–530 μm thickness, 2 nm roughness). Mattos et al. (Mattos et al. 2019) spin coated a 4 nm thick layer of either gold, or platinum/palladium alloy, or iridium on freshly cleaved mica discs. Using this technique nano-scale features can be resolved as shown in Fig. 8c. The high contrast of NegC-SEM images facilitates CNF image analysis, while retaining the resolution to image nano-sized features.

SEM image capture requirements

To obtain accurate and meaningful data when using SEM to image CNFs, several factors must be considered:

- (1) Purpose-Specific Magnifications: The measurement of fibril width is dependent on the magnification of the image. Various magnifications may be necessary to capture both the overall structure and detailed features of the CNFs. Low magnification is useful for assessing larger features, such as the entire size of the CNF object and the dimensions of larger fibril bundles, while higher magnification at multiple locations is necessary to have sufficient resolution to assess the widths of individual fibril branches (Fig. 9). The magnification range required may be reduced if larger fibers and fragments have been separated prior to imaging.
- (2) Feature Identification: It is crucial to identify specific CNF features to be analyzed, such as the level of branching, the position to measure

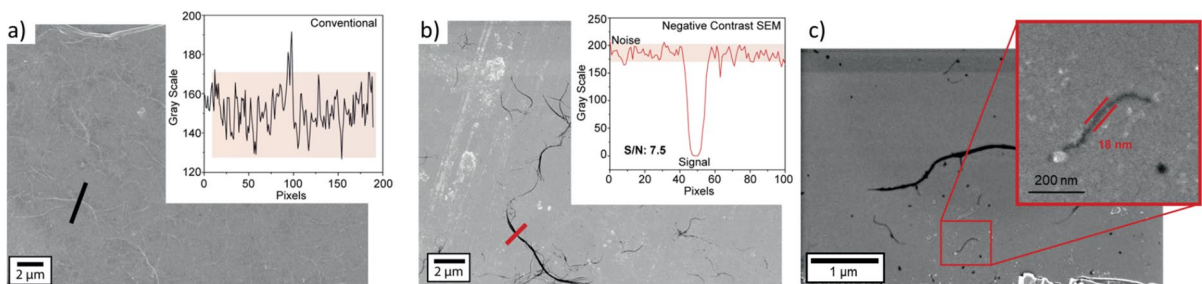


Fig. 8 Comparison in image contrast between conventional and negative contrast SEM imaging of CNFs. **a** conventional SEM sample configuration with CNFs first deposited on SEM stub, then subsequently coated with a thin conductive metal coating. **b** and **c** NegC-SEM sample configuration with CNFs deposited on a highly conductive substrate. The

inserts in **a** and **b** highlight the higher contrast for the NegC-SEM imaging, while the insert in **c** highlight the resolution of NegC-SEM. All images were obtained using the same SEM [FE-SEM, Zeiss Sigma VP] and imaging conditions [1.5 kV, working distance of 6 mm], with an in-lens detector. Adapted from (Mattos et al. 2019)

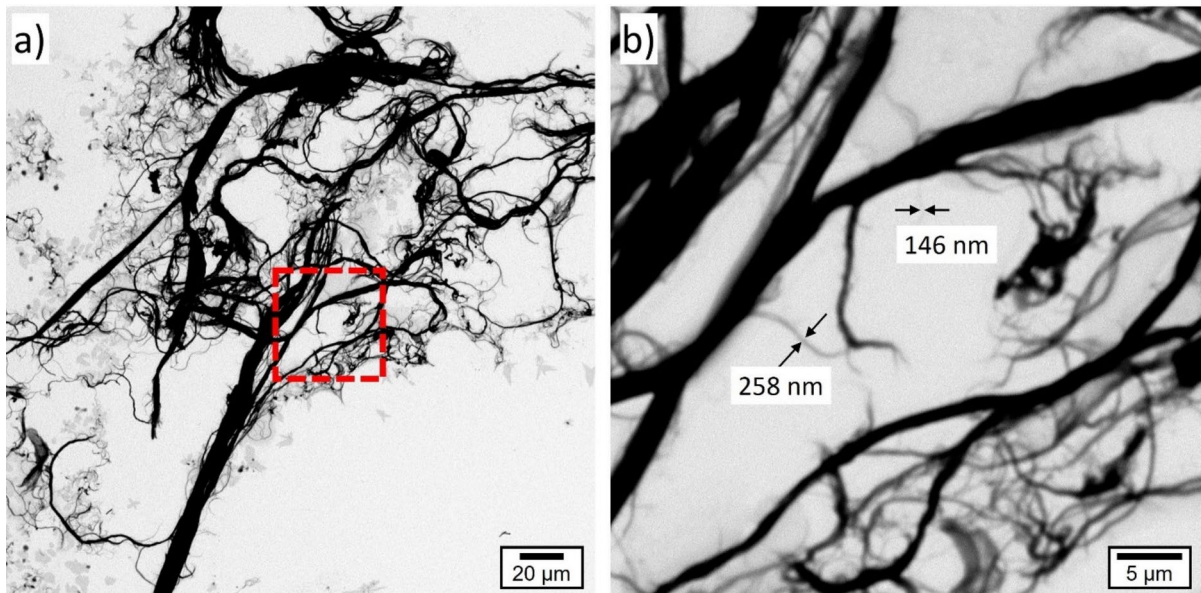


Fig. 9 Negative contrast SEM of a CNF particle. **a** lower magnification, and **b** higher magnification of the red boxed region in part a. By using two different magnifications different sized features within the CNF particle can be measured. Sample preparation: 2 μL of a 0.001 wt% CNF suspension was deposited onto a silicon wafer (2 nm roughness), and air dried for at

least 12 h before SEM imaging. Imaging was completed using a benchtop SEM (Phenom Pure), operating at 5 kV, working distance of 8.8 to 8.9 mm, and backscattering detector. Images were adapted from (Ringania et al. 2022). Reproduced with permission from Springer Nature

branch width and width distributions across these levels.

- (3) **Image Capture Locations:** Determining where to take images is vital for consistency, accuracy, and avoiding inadvertent selection bias. Multiple areas should be imaged to ensure a representative sampling of possible morphologies, avoiding the tendency to image only isolated or simple objects that may be less challenging for particle size analysis. Ideally, the same object should only be imaged once at each magnification to avoid redundancy and ensure a comprehensive analysis. Realistically, for large CNF objects it may be necessary to overlap multiple images to have a final analysis of all fibril widths, but one has to take care that each feature/branch is counted only once for statistics. The number of images must be sufficient to give a representative view of the CNF objects which will depend on the sample morphology.
- (4) **Magnification Calibration & Corrections:** When using various magnification levels for size analysis, it is important to ensure proper instru-

ment calibration across the magnifications used. When performing statistical characterization, it is important to connect the findings to the correct magnification to ensure accurate measurements and comparisons. Proper calibration and correction for magnification discrepancies are essential for reliable data interpretation.

Manual image analysis

There are a number of factors that should be considered when determining width and length distributions from electron microscopy images. These include the number of particles that should be measured, whether it is important to measure all particles in an image versus a representative number, how/where the width and length should be determined, whether the sample preparation method leads to variation in the measured dimensions, whether both length and width can be measured from the same image or whether multiple image scales are needed and, finally, how the data are presented/reported.

These factors need to be considered regardless of whether the analysis is manual or automated. Most papers that have used EM (or AFM) to measure particle size distributions for cellulose nanomaterials have used manual image analysis, occasionally with the microscope software but more routinely using custom software or freely available software such as ImageJ. Some recent papers have used some of the automated features available in software or custom macros to help to streamline the analysis process, but still rely on manual selection and analysis of individual particles (Bushell et al. 2021; Meija et al. 2020).

General guidance on a number of factors, such as how the required particle number varies with the level of uncertainty that is acceptable and the breadth of the particle size distribution, can be found in (ISO 2021b) which focuses mainly on inorganic nanomaterials. However, the typical high polydispersity, high aspect ratio and strong tendency to agglomerate and aggregate make image analysis a considerably more challenging task for CNFs. Another important factor is the significant level of branching that is typical of most CNF samples. In principle one should measure each individual fibril, but for a branched structure it is unclear whether one should measure a branch only once, independent of the number of interconnections, or measure the branch width of the fibril between all the interconnections. A summary of the approaches that several groups have used illustrates both the issues and potential solutions.

With high levels of branching and entanglement, it is often difficult to measure discrete CNF objects. Accordingly, most researchers report an average of measured nanofiber segments, whether part of a larger object or stand-alone. In many cases the entanglement makes it almost impossible to tell when one branched structure ends and another begins. The number of such CNF fibrillar elements that have been reported have varied widely, including not being reported at all. Some numbers, where reported, from the literature are e.g., 100 CNFs (Berglund et al. 2016; Pyrgiotakis et al. 2018); > 120 CNFs (Zhang et al. 2012, 2015), at least 150 CNFs (Ang et al. 2020), 200 CNFs (Mattos et al. 2019) or several hundred CNFs (Ringania et al. 2022). Ang et al. (Ang et al. 2020) did a statistical analysis of median width with an increasing particle count and thus recommend analysis of 150 CNFs for their material, provided that all objects in the selected area are measured. Note that both the

sample morphology and polydispersity and the level of acceptable uncertainty will determine how many CNFs need to be analyzed for a representative measurement for a specific sample. If high accuracy is needed, then one should use a similar approach to the Ang et al. paper to confirm the number of CNFs that must be measured to obtain a stable median.

A more general recommendation is to measure at least 200 fibrillar elements in total from multiple images in order to capture the sample heterogeneity. Note that a highly branched CNF may consist of relatively large structures with many interconnections between branches, but could still be considered as a single particle. In such samples it is important to specify exactly how the measurements are done, to sample as many “branches” as possible in each image, and to ensure that one measures dimensions of multiple large branched structures. If the size measurements are required for regulatory purposes, it is crucial to understand the regulator’s expectations for how the dimensions are to be assessed—or to work with them to decide on a reasonable approach.

The selection of particles to be measured is important. Ang et al. (Ang et al. 2020) studied the factor of potential operator bias. The results showed that when the operators were asked to select fibers, they showed consistent bias towards larger fibers thus rendering the results unreliable. It is therefore recommended to measure all the fibrils within each analyzed image or selected area within an image. However, note that one should not analyze fibrils touching the edge of the image or area. Furthermore, it may not be possible to measure all particles in the image for samples with highly branched and interconnected CNF. It is also useful to provide one or more annotated images that illustrate how the analysis has been done, as in recent studies (Ang et al. 2020) and (Ringania et al. 2022).

For a limited set of samples, width and length determination can be done in the same image with a given magnification. Typically, different images with different magnification are needed. Pyrgiotakis et al. (Pyrgiotakis et al. 2018) used an approach where images were taken at different magnifications and the diameter of each fiber was measured at a minimum of five points along the fibril length; the length was traced from end to end of each fiber. Ringania et al. (Ringania et al. 2022) used three different magnifications to capture different sized CNF fibrillar elements. Mattos et al. (Mattos et al. 2019) measured the width

at the midpoint in length. For branched structures one has to decide whether to measure branches at multiple locations between individual interconnections. Also note that SEMs with lower resolution may not be able to resolve the smallest fibril branches in which case TEM may be necessary (Pyrgiotakis et al. 2018; Rol et al. 2019; Wang et al. 2012).

Most examples that have determined particle size distributions have focused on width measurements as it is typically challenging to measure length for most CNF samples. However, for samples for which aspect ratio is an important parameter, it is necessary to measure length and width of the same CNF. This will require collection of images on two (or more) magnifications in order to measure the length of longer particles as well as the width of smaller particles. This should be done by measuring smaller images within a large image to ensure that accurate values for both width and length are obtained. Alternatively, one can use methods such as sedimentation (Raj et al. 2016) or rheology (Cainglet et al. 2023) to obtain an aspect ratio and combine this data with width measurements to obtain the CNF length.

Network and branching are common features of CNF material. When the intent is to measure the dimensions of CNF objects and their fibril elements, it is highly recommended to work with dilute samples and substrates that minimize fibril entanglements and network formation thus facilitating identification and measurement of both the CNF objects and their fibril elements. If that is not possible, special considerations have to be made in image analysis. A few attempts to quantify CNF branching have been reported, e.g. by (Pyrgiotakis et al. 2018), albeit on TEM images. They introduced two parameters to describe a network-like structure. One was the node-to-node distance, defined as the distance between the centers of two nodes; values of 340 ± 230 nm and 570 ± 515 nm were measured for CNF samples of nominal width 50 and 80 nm. The second parameter was the number of individual fibrils per node which was similar for the two samples (~ 3.2 fibrils per node).

Branching is, just as network structuring and interconnected fibrils, another important factor which is not yet commonly addressed in the literature. Ringania et al. (Ringania et al. 2022) measured the fibril widths at each branch but without measuring the same branch at multiple points. This approach would give relatively high impact to the average or median

width from the small branches and relatively small impact from the thick, often micro-sized, fibril bundles. Depending on the purpose of assessing CNF dimensions, it may be useful to distinguish between measurements for small individual fibrils and larger branched structures.

The statistical treatment to be utilized can depend on the purpose of the measurement. Ang et al. (Ang et al. 2020) and Mattos et al. (Mattos et al. 2019) reported the median width rather than the average width, since the median is generally a better measure of central tendency for samples with an asymmetric particle size distribution. Histograms were also reported in both publications. Note that for regulatory purposes one may require particle size distributions based on either the number fraction or mass fraction of particles that have nanoscale dimensions. A mass-based average would be challenging to obtain by SEM and may require assessment of the volume fraction of CNFs in the nano range, which seems challenging for branched structures. A number average would be more straightforward to obtain from a size histogram, but the histogram itself is still expected to be challenging to obtain for branched CNF structures. When reporting length or width data obtained it is recommended to describe how all the factors identified in bold text above have been handled, at least until an established standard is available.

Automated image analysis

Automated analysis of SEM images would be desirable due to the large number of objects that must be analyzed. Two software packages that have been used for image analysis of CNMs in the literature are ImageJ (Mattos et al. 2019; Meija et al. 2020) for EM and Gwyddion (Bushell et al. 2021; Mattos et al. 2019) for AFM. Both are open source and freely available. They have a wide variety of capabilities and can in principle be used for automated analysis. So far there are very few examples of thresholding to identify CNM particles and automated analysis of their dimensional parameters. Examples are primarily for either CNCs or CNFs and include use of FiberApp (Bushell et al. 2021; Usov and Mezzenga 2015), a custom software package designed for analysis of fibers, polymers and biomacromolecules, and a custom Python package (Willhammar et al. 2021). When combined with image processing algorithms,

FiberApp has been used to assess length, widths, orientation of various polymer fibrils (Persson et al. 2017), and may be applicable to assessing the fibrillar elements of CNF objects. Other software, such as DiameterJ and DNA Trace, can in principle also measure height and length, respectively, for fibrils such as CNFs (Hotaling et al. 2015; Mikhaylov et al. 2013).

To date the most satisfactory approach for automation for CNM analysis is a semi-automatic comprehensive image processing system, SMART (Standardized Morphology Analysis for Research and Technology) for analysis of both TEM and AFM images of CNCs. The software uses a multi-step approach with pre-processing to enhance image quality, grouping of features to identify specific classes of CNCs (border, isolated, aggregated) and algorithms for digital measurement of CNC dimensions. SMART was initially tested on a CNC reference material (Yucel et al. 2021) and particle-by-particle analysis of manual and SMART analyses demonstrated that TEM and AFM length and width (height) distributions were similar for the two approaches, although there was a slight narrowing of the TEM width distribution with SMART. The study highlighted the importance of starting with good quality images for a reliable SMART analysis (Yucel et al. 2022).

The SMART approach has the potential to significantly decrease the time required for analysis and may remove some of the analyst subjectivity in identifying individual particles. It may also be useful for assessing the number of objects needed for a stable dimensional measurement. Current work is directed towards extending SMART to the analysis of CNF, a much more challenging problem, given the level of interconnectedness and branching typically observed for these materials.

Recommendations for testing and reporting results

1. *Sample information* Provide as much information as possible on the CNF sample used, including the source of cellulose, preparation method, any surface or other modifications, any fractionation before imaging.
2. *Pre-Analysis* Before imaging, it is recommended to perform an initial characterization of the sam-

ple with a fiber analyzer. Critical points to establish include whether there are large fibers within the sample and some indication of the size distribution of material. To facilitate analysis of the smaller objects in heterogeneous samples, it is particularly recommended to fractionate the sample to separate the larger and smaller objects. The use of these techniques will allow SEM measurements to be used to analyze the fraction of fibrils with diameter 10–100 nm, which are difficult to analyze by other techniques. We note that despite many advantages, such preliminary analysis is relatively rarely reported in the literature.

3. *Sample preparation* Sample concentration and preparation conditions should be chosen to produce a layer of fibrils with some spacing between, while minimizing fibril entanglement. The exact weight fraction will be of the order of 0.001% but will depend on magnification and sample conditions and the object of the measurement. We recommend at least 5% coverage to achieve reasonable numbers of fibrils per image. Achieving these conditions may require an iterative process. It is essential that all the steps used in preparing the sample be reported, as the results can significantly depend on the techniques used. We recommend reporting the:
 - (a) Concentration of CNFs and solvent used to prepare the sample and any pretreatment used (vortexing, sonication, etc., with details on instrument used, power/energy, time);
 - (b) Substrate and pretreatment (e.g., plasma cleaning of grids, precleaning of silicon chips (e.g., ozone), polymer coating, metal coating)—include details (concentration, time, etc.) on how any pretreatment is done
 - (c) Amount/concentration of sample used and how it is deposited (drop cast, dipped in solution, spin-coated, etc.), time, rinse if used, drying method
 - (d) Coating of final sample for good SEM contrast including reporting the metal and instrument used, as well as coating parameters such as time and pressure etc. The final thickness deposited, if this is available, should also be reported.

- (e) Number of samples prepared and any variations in procedure to test various parameters
4. *Imaging* The system used should preferably be of the FE(G) tungsten cathode type. For automatic or semi-automatic image analysis, it is recommended to have high contrast images. One method to increase contrast between the CNFs and substrate is to use NegC-SEM. Images should contain a sufficient number of objects (see below) that do not touch the image edge. For some highly processed samples it may be feasible to measure the fibril length and the width and length measurements will often require different magnifications. We recommend reporting:
- System type, cathode type, and detector type (e.g., lateral secondary electron, in-lens secondary electron, backscattered electron)
 - Settings: accelerating voltage, working distance, and the magnifications used
 - Approach used for image selection to ensure a representative, non-biased description of the CNFs.
5. *Analysis* The number of objects to analyze needs to be decided depending on the purpose of the analysis and the character of the material. A general recommendation is at least 200 objects. All of the objects in the images or a selected area that do not touch any image edges should be analyzed and if there is branching, measure each branch. We recommend reporting:
- Number of objects analyzed and how they were selected (recommended: all objects not touching an image edge).
 - Explanation of where on the objects the width or length was measured, preferably with an annotated image.
 - How the branching and possible network formation was handled in the analysis.
 - Whether manual, semi-automatic or automatic analysis was used and how it was performed.
 - A histogram of the size distribution and selected statistics such as average, median and appropriate numbers that describe the width of the distribution.

Conclusion and path forward

The dimensions of CNF objects should be measured to ensure consistency in production, suitability for an application and to meet regulatory requirements. At the same time, the complexity of CNF objects with their branching structure and multi-scale object features makes it difficult to measure their dimensions in a reliable and reproducible way. This paper has reviewed the techniques available for the measurement of CNF object dimension, focusing on SEM measurement of CNF object dimensions. In comparison to other techniques, SEM is shown to be relatively quick and to be able to characterize CNF objects at a variety of length scales from microns to nanometers.

From our analysis of the best practices in the literature and personal experience we have developed a set of recommendations for the measurement of length and width of complex CNF objects, covering initial characterization, sample preparation, imaging and image analysis and reporting. It is our expectation that measurements following these protocols will provide a much stronger platform to meet production, characterization and regulatory requirements.

It is acknowledged that there are still outstanding questions on how best to do the measurement and analysis. A good dispersion technique would be needed to avoid agglomeration and entanglement as much as possible. It would be good to have a comprehensible recommendation guide on different dispersion techniques related to different CNF morphology. When measuring especially the width, it is needed to know how to select where to measure on the branched objects and how many points to be measured per object. The points could be equidistant or just one per branch. This, in its turn, may need research on how different amount of branching affects the material properties. Is the number of branches more important than the width of them? We challenge the CNF research community to explore these questions.

To answer these questions will require development of an initial protocol for imaging and analysis and its testing in several laboratories. Such a study will require the use of the same samples in different labs and will benefit from use of reasonably well-behaved samples that are compatible with SEM imaging. A reliable dispersion method will also have to be developed. Finally, it will be important to specify how size analysis should be done, with particular emphasis on how and where to measure

fibril width, how to describe branching and whether length is also required. Results of such a mini-Inter Lab Comparison (ILC) can be used to optimize the protocols for both imaging and analysis, carrying out additional tests or examining additional samples as needed, prior to initiating a full ILC with a larger number of participants. It would be beneficial to run the full ILC in two stages with the first phase testing only the image analysis method using one or two image sets recorded by one lab for the samples to be used for phase 2. This will better test the suitability of the analysis protocol, which can be further optimized if needed. Finally, all participants will measure several CNF samples, and analyze the data and submit results, following the specified protocol to the best of their ability. The protocol and validation data from the full ILC can then form the basis for an ISO standard, possibly written concurrently with the later phase of the ILC.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval Not applicable.

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