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Metrology and nano-mechanical tests for Nano-Manufacturing and Nano-Bio Interface: Challenges & Future Perspectives

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ABSTRACT

Nanometrology refers to measurement techniques that assess materials properties at the nanoscale. Laboratory-based characterization of nanomaterials has been the key enabler in the growth of nanotechnology and nano-enabled products. Due to the small size involved, dimensional measurements has dominated such characterization underpinned by a tremendous development in stand-alone electron/ion microscopes and scanning probe microscopes.

However, the scope of nanometrology extends far beyond off-site, laboratory-based measurements of dimensions only, and is expected to have a tremendous impact on design of nano-enabled materials and devices.

In this article, we discuss some of the available techniques for laboratory-based characterization of mechanical and interfacial properties for nanometrology. We also provide a deep insight into the emerging techniques in measuring these properties, keeping in view the need in advanced manufacturing and nanobio-interactions to develop multifunctional instrumentation, traceable and standardized methods, and modelling tools for unambiguous data interpretation.

We also discuss the evaluation of nanomechanical properties and surface/interface response of materials, within the purview of manufacturing processes and standardization.

Finally, we discuss scientific and technological challenges that are required to move towards real-time nano-characterisation for rapid, reliable, repeatable and predictive metrology to underpin upscaling nanomaterials and nano-enabled products from the research field to industry and market.
1. Introduction

Nanometrology is a sub-discipline of metrology and is concerned with the science of measurement at the nanoscale level including the quantitative determination of dimensions as well as other physical properties e.g. mechanical, electrical, magnetic, optical properties and combination thereof, chemical and biological properties of nanomaterials and events taking place at the nanoscale [1-3]. The magnitude as well as the tolerance and uncertainties in the measured values are an essential part of metrology.

Measurements in the nanometre range should be traceable, using internationally accepted units of measurement (e.g. of length, angle, quantity of matter, displacement and force). This requires common, validated measurement methods, calibrated scientific instrumentation as well as qualified certified samples. Nanometrology is emerging as a new strand in metrology aiming to development and use tools and methodologies for the measurement and characterization of structures and materials with tailored properties at the nanoscale.

Advancing nanometrology is an urgent requirement today to ensure successful convergence of nanoscience to useful products and processes into the market place [4]. Despite a tremendous scientific and technological progress in the field of nanomaterials and nanotechnology, new techniques, tools, instruments and infrastructure are critical to support high-performance, cost-effective, reliable instrumentation and improved measurement methods in nanometrology.

A specific and well-defined need exists for designing a technological roadmap to address the current and emerging challenges in this field. Such needs include the development of new reference materials, calibration methods, and accurate predictive modelling tools alongside reliable, rapid, and integrated instrumentation. The integration and coupling of multifunctional measurement techniques underpinned by modelling at multiple length scales
is a key determinant in the upscaling of nanotechnology and nanomaterials into the industrial market.

Extensive data available on function and performance of nanostructured and nano-enabled materials is of limited value if these materials are not well characterized, and the measurement methods and equipment traceability are ill-defined. When these materials are translated into products, a general agreement on measurements of the constituent nanomaterials and a clear link to the desired functional performance are needed. For example, if a product contains nanomaterials, the size, shape and the volume fraction of nanomaterials in the product must be defined unambiguously and the enhanced performance of the product enabled by the nanomaterial should be known. The achievement of such definition and standardization calls for a significant effort in the fields of, for example:

- High resolution (HR) three dimensional (3D) characterization of, especially, embedded nanostructures
- Speed of characterization
- Real-time (in situ and/or lab-scale) characterization
- Interface characterization
- Quantitative measurement of dispersion of nanoscale materials within a matrix
- Modeling and simulation
- Standardized interpretation of data.
- Advanced tools for data analysis/storage/sharing

The ability to image with a nanoscale resolution and to reveal the dimension and chemistry of the constituents and the resulting structure, morphology, mechanical, chemical and interfacial properties at such spatial and depth resolutions has become the cornerstone of the research and development (R&D) in nanoscience and nanotechnologies. Such ability addresses themes relevant across a number of manufacturing [5-7] and processing sectors: the semiconductor industry, mechanical engineering, life sciences, molecular electronics, biosensors, biomaterials and nano-bio interfaces just to name but a few. The scale of such interest is
reflected in the size of the global market for molecular, atomic and nanoscale imaging, which is expected to grow from nearly $3.1 billion in 2011 to $4.4 billion by 2017 [8]. The dominant application area is biological sciences (39%) and materials science (33%), which, in 2011, represented over two-third of the market. This is notwithstanding the fact that most of the current microscopy applications in these two fields are related to R&D rather than to production. Microscopic, or more specifically, nanoscopic imaging has surely become one of the favoured tools to aid the dimensional, morphological and structural measurements of nano and biomaterials, including characterisation of industrially relevant materials and its implication for design of innovative products. In this context, the need to develop high-speed and real-time metrology tools is becoming more and more important for the design of nano-materials and nano-enabled devices, since they were systematically introduced into commercial products.

Among a very wide range of possible nanometrology tools available, those based on the use of mechanical contact probes (particularly, AFM and nanoindentation) and chemical imaging possess the potential to become effective tools to make nano-materials design and production more effective, given their possibilities of high-speed/high-throughput reliable and low-noise testing. Chemical imaging is a powerful that can also be customised for on and in line analysis in a number of process and manufacturing sectors including food, agriculture, pharmaceutics, recycling, forensics, and medicine. Of particular interest is the general applicability of spectroscopic tools for both biological and nonbiological materials.

This article thus critically reviews salient features of current and emerging imaging/contact-based nanometrology that provides quantitative measurements of surface mechanical and chemical properties of nanomaterials and nano-enabled products. Specific emphases have been given on the potential impact that such techniques may have on the design and characterisation of hybrid materials where hard/soft interfaces play a key-role in determining
mechanical/chemical properties and in-service behaviour (e.g. nano-structured composites, biomaterials, engineered scaffolds, etc.). Additionally, the issue of the establishment of nano-scale metrology characterisation techniques as effective tools for in-line and real-time characterisation of industrial products is analysed and discussed. Finally, we identify critical gaps and challenges in this field and identify means that potentially overcome these gaps and challenges to realise the translation of nanometrology towards industrial practice.

2. Current state-of-the-art in mechanical nanometrology by nanoindentation

Mechanical nanometrology is referring among others to the mechanical characterization of nanostructures, nanostructured materials and bulk materials at the nanoscale [9]. Instrumentation and metrology for nanomechanics consist of a variety of measurement techniques, the target of which is to assess the mechanical behaviour of materials.

Among these techniques, nanoindentation has emerged as a convenient and fast method for the assessment of localized surface mechanical properties. In nanoindentation, a diamond indenter with known geometry is pushed into a specimen surface while the applied force and the resulting displacement values are recorded simultaneously and loop-controlled through actuators and sensors (Fig. 1). The hardness (measured as the resistance to contact pressure) and the elastic modulus of the material being tested is determined from the first segment of the unloading curve assuming an elastic behaviour using the theory of contact mechanics [10-12]. The standard method is capable of providing information on the elastic recovery and plastic deformation [11], with unprecedented lateral and sub-surface depth resolution. Additionally, nanoindentation testing can also be used in measuring mechanical properties such as fracture behaviour [13-18], hardening, strain rate sensitivity, residual stress [15, 19-21] and size effects in plasticity on a local level [18, 22-28].
Figure 1. Comparison of the load-displacement curves obtained from nanoindentation experiments performed at the maximum loads ranging from 1,000 to 7,000 mN [4].

Key-issues in nanoindentation experiments have been analysed in detail and reviewed in several papers during the last two decades: experimental inaccuracies and related measurement errors can be associated to (a) uncertainties in surface determination (very critical for very soft/compliant materials), (b) time-dependent sample behaviour (a critical issue for soft polymers and biological tissue), (c) substrate effects during thin film testing, (d) pile-up induced errors, (e) microstructural and size effects that affects collective response of the material.

In addition to this, new challenges have been grown in the last few years, as a direct consequence of the fast development of nanomaterials and micro/nano-devices.

A first consequence of miniaturisation of components is the requirement, from the design to processing of components, to take into account scale effects in mechanical behaviour of materials, with specific focus on plasticity and fracture at the micro/nano-scales.

Starting from the pioneering work by Uchic and co-workers [29], size effects in plasticity of metals have been extensively used by pillar testing, an experimental technique that involve uniaxial compression of micro/nano-pillars (fabricated by several techniques, including FIB) by nanoindentation testing, usually equipped with a flat-punch indenter. As reviewed in recent papers [18], the knowledge developed show a complex framework where the strength of
crystalline materials at small scales is shown to be remarkably affected by the initial microstructure of the material (grain size, dislocation density,…), with remarkable differences as a function of crystal structure (fcc, bcc, hcp,…). Additionally, testing of small scale structures usually involve the transition of the deformation mechanisms towards a highly stochastic behaviour with the presence of discrete strain burst in the stress-strain curves.

As a direct consequence, size has become an important design parameter for nano-technology and micro-device industry, where the changes in strength with miniaturisation cannot be discarded anymore and should be fully considered for the design of novel nano-enabled materials.

In parallel, the fracture and interfacial behaviour of nanomaterials and micro-device has recently established as a hot topic in the nanomechanical materials testing community [30]. Thanks to the establishment of micro-fabrication routes and in-situ nanomechanical testing devices, a novel series of experimental techniques has been recently developed and optimised, which allowed scientists to gain further insight into the microstructural and size effects on fracture toughness at the micro and nano scales [31]. Several experimental methodologies for micro-scale fracture mechanics have been recently proposed, namely the cantilever bending, double-cantilever bending and pillar splitting, allowing for quantitative fracture toughness measurement on micron-size specimens, as well as accurate estimation of interface toughness in hybrids and composites.

Here, the combination between nanoindentation testing with high-resolution analytical tools (e.g. SEM, nano-diffraction, micro-Raman, etc…) gives additional dimensions to nanomechanical testing, which has been practically evolved into a real mechanical microscopy. In particular, in-situ nanoindentation has already been extensively used for the assessment of mechanical behaviour and failure modes of 3D printed nano-architectured materials, obtained by Direct laser Writing (DLW) lithography [32].
Even more recently, the space of nano-scale mechanical testing has been expanded towards the temperature domain. Novel high-temperature nanoindentation systems (including in-situ SEM devices) have been developed and validated, thus allowing for an accurate quantitative assessment of temperature dependent deformation mechanisms in nano-enabled materials, coatings, hybrids and composites.

Despite the fact that tremendous efforts have been put into the development of novel nanomechanical testing methodologies, some major lacks are still present to (a) improve the reliability and reproducibility of the methods, (b) improve the accessibility of such methods to industrial applications for reliable testing on real products, and (c) improve the speed of characterisation in order to fill the industrial demand for high-throughput reliable characterisation methods.

Since industrial applications demands for a quick and effective upgrade of such advanced technologies to in-line and real-time testing, it is clear that current main challenges are represented by the improvement of:

(a) The speed of nano-mechanical characterization, including data acquisition speed;

(b) Its capabilities for dynamic testing with high-frequency data investigation;

(c) Its capabilities of testing interfaces of highly heterogeneous materials;

(d) Rapid mechanical properties mapping over large areas in composites and hybrids.

In the following chapters, we will introduce some novel advances in the fields of nanoindentation procedures that attempt to overcome those limitations. In particular, we will show that the use of high-speed and high frequency nanoindentation can represent a breakthrough for advanced and fast characterisation of strongly heterogeneous materials, as well as for promoting the use of nanoindentation as a tool for rapid prototyping and surface nano-patterning.
Table 1 A summary with references of the latest developments, materials insights and “implications for Design” of nanoindentation-based characterisation methods

<table>
<thead>
<tr>
<th>Technique:</th>
<th>Materials Insights:</th>
<th>Implications and Impact for Design of:</th>
<th>Refs.:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro-pillar testing</td>
<td>Effects of specimen’s size and microstructure on strength and plasticity.</td>
<td>Mechanical behaviour of MEMS and other miniaturized components. Mechanical behaviour of nano-architected materials and nano-structures produced by 3D printing.</td>
<td>[23-29]</td>
</tr>
<tr>
<td>Micro-scale fracture experiments</td>
<td>Fracture toughness, failure mechanisms; Size and microstructure effects on fracture toughness.</td>
<td>Influences of size and microstructure on fracture toughness of nano-enabled materials, microelectronics devices, battery composites and multi-layered coatings.</td>
<td>[13-17]</td>
</tr>
<tr>
<td>In-situ nanomechanical testing (including tension/compression/bending/delamination/debonding)</td>
<td>Nano-scale deformation and failure mechanisms of nanomaterials; correlation between microstructural evolution and mechanical behaviour.</td>
<td>Support for microstructural design of multi-phase alloys. Design of MEMS device with multiple layers. In-situ assessment of the mechanical behaviour of 3D printed nano-architected materials. Mechanical behaviour of nanoparticles, nanowires and nanotubes.</td>
<td>[18, 24, 26, 30, 36]</td>
</tr>
<tr>
<td>Nanoindentation mapping (or statistical nanoindentation)</td>
<td>Distribution of mechanical phases within a multi-phase and/or hybrid material.</td>
<td>Design of novel hybrid materials and composites (battery materials, cements, fibre-reinforced composites, bio-interfaces, biological tissue).</td>
<td>[37-42]</td>
</tr>
<tr>
<td>High-Temperature nanoindentation</td>
<td>Analysis of thermally activated deformation processes, creep and their correlation with microstructures.</td>
<td>Design of novel materials for high-temperature and energy applications. Studies of brittle-ductile transition on a small scale.</td>
<td>[14, 43-47]</td>
</tr>
</tbody>
</table>
3. Current state-of-the-art in mechanical nanometrology by atomic force microscopy (AFM)

Scanning probe microscopy (SPM) and atomic force microscopy (AFM) systems are capable of measuring mechanical properties at the nanoscale by using custom-designed probe tips. Erroneous use of SPM may however lead to tip damage and consequently blurred imaging (Fig. 2). SPM systems have been widely developed and rendered as essential tools not only for the investigation of mechanical properties of small volume of nano-based materials but also for the assessment of key surface properties and deformation (Table 1). However, their ability to measure at the nanoscale is still limited to mostly qualitative data obtained through recording via image scanning of the plastic deformation and roughness. The main obstacles include the lack of knowledge of the actual interaction between the probe and the surface (e.g. Van der Waals-electrostatic forces), the uncertainties in the real geometric features of the probes, which could change significantly between measurements, the non-
linear performance of the piezoelectric scanners, and the lateral motion of the cantilever probe.

SPM uses a sharp probe, which is scanned over the surface of a material being imaged. The technique is capable of providing atomic scale resolution routinely on electrically conductive, semi-conductive or insulator samples. While electron tunnelling can occur in biological species, the presence of water makes conductive SPM such as scanning tunnelling microscopy (STM) of biological samples difficult due to charge leaking and shunting. At the ambient and in liquids, lateral resolution is slightly lower for atomic force microscopy (AFM) and its numerous variants compared to what can be achieved if the imaging were carried out in vacuum or in dry conditions. A 15-30 nm resolution is possible in most commercial instruments and using commercial probes. A plethora of information can be extracted from such techniques for example: topography, adhesion, electrostatic force, surface potential, piezoelectricity, spreading resistance, impedance, electrochemical potential and so on.

Another mode of SPM, scanning near field optical microscopy (SNOM) involves holding and scanning a nanoscale-sharp tip or optical fibre above the sample surface and measuring the fraction of the optical signal influenced by the tip presence. This technique can be used for super-resolution (i.e. beyond the diffraction limit of optical imaging) vibrational spectroscopy of materials, which can also be used to extract mechanical and structural information as well as chemical properties of materials at high dimensional resolution.
Table 2. SPM imaging to obtain key materials properties for application

<table>
<thead>
<tr>
<th>Sample</th>
<th>SPM image</th>
<th>Edited image</th>
<th>Property</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thin films deposited by HiPIMS</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td>Deformation, pile-up, roughness</td>
<td>[33]</td>
</tr>
<tr>
<td>NiO: Au hydrogen sensors</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td>Pile-up</td>
<td>[48]</td>
</tr>
<tr>
<td>Powder coatings</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td>Adhesion, Pile-up</td>
<td>[49]</td>
</tr>
<tr>
<td>CNT composite</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td>CNT dispersion</td>
<td>[50]</td>
</tr>
<tr>
<td>FGM Cu-alumina</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td>Agglomeration, phase identification, roughness</td>
<td>[51]</td>
</tr>
</tbody>
</table>

AFM systems have demonstrated success in mechanical testing at a variety of low loads ranging from µN down to nN range albeit with significant limitation in load applications due to tip and cantilever. Operating under a close control of displacement and force measurements
that are inherent in AFM systems, the technique can evaluate nanomechanical properties of surfaces at high spatial resolution. These systems can also be used to produce lateral force or friction force maps that derive their measurements from the torsion (i.e. the lateral twist) of the cantilever. Still, there are many accuracy issues e.g. inadequate traceability and calibration associated with the measurement of the mechanical integrity by AFM. Tip size evaluation is a particular contributing factor in such measurements as the tip size is extremely small and thus difficulties are originated from the current needs for accurate and traceable low force measurements and topography imaging (Figure 3) [37].

![AFM images of a CNT forest in 3D and from top view (field of view: 5 × 5 μm²), where the edges of carbon nanotubes (CNTs) forest surface have been observed [37].](image)

**Figure 3.** AFM images of a CNT forest in 3D and from top view (field of view: 5 × 5 μm²), where the edges of carbon nanotubes (CNTs) forest surface have been observed [37].

Nano-scratch tests are also important in the fundamental understanding of interfacial phenomena at small scale by studying parameters such as surface roughness, adhesion, friction coefficient, scratch and wear and relevant mechanisms (e.g. ploughing). Nanotribological studies can be conducted by both nanoindentation and AFM.

For both of these techniques major limitations arise from the determination of the real contact area and the lateral resistance which is related to higher rigidity requirements. The accurate calibration of the lateral force and control of low magnitude forces and displacements should also be considered. Another key issue is the vertical penetration during sliding of the tip, since it can be controlled only up to a few nanometers [52].
Figure 4. SPM images (field of view: $10 \times 10 \, \mu m^2$) of nanoindentation imprint in the composite coating (top left and right) and interface identification through contact pressure (hardness) for composite coating (sandwich)(FC, Teflon® and plain TEOS individual testing was needed) [36]
4. State-of-the-art Imaging of Nano-biointerface

Imaging nanobio interface can become a cornerstone in the metrology of bio-nanotechnology (or nanobiometry) similar to what electron microscopy was for the drive towards miniaturization in semiconductor industry. Nondestructive tests of biological building blocks, predictions of antimicrobial or anti-biofilm performances, cellular pathways of drugs and nanoparticles - all requires metrological solutions although there may not be any ‘one size fits all’ type solution. The need for solutions however is becoming urgent due to the emergence of electronic DNA sequencing, single molecule mass spectrometry, antimicrobial wound dressings, socks and textiles, drug coated stents and nanomedicinal products such as those for cancer therapy which are either in the market, near market or in clinical trial stage. This long-standing need for the understanding of the interactions between biological systems and artificial nanostructures[53]arises from the needing to address issues such as those listed below [54]:

i) The design of nanostructures to prevent biological degradation of therapy through systemic pathway towards the target treatment sites

ii) The absorption of nanostructures by cells,

iii) The uptake and recycling of nanostructures in biological systems,

iv) Trans-endocytosis and Endosomal escapes of nanostructures, and

vi) Nano-safety evaluations.

Addressing the above requires nanoscale measurements in ambient conditions often in the presence of water and at the nano-bio interface. For nano-bio products, it is also critical to ascertain their production reliability, repeatability and biological performance without adding a large cost for quality assurance and control. From both compatibility and cost point of view,
most vacuum based measurement techniques are not practical metrological tools for nano-bio products and production.

The detection, characterisation and quantification of inorganic engineered materials has been widely discussed in the literature, please see ref.[55] for a recent review on this topic especially in relation to the techniques and methodological approaches for the analysis of nanomaterials within complex samples. The general methods currently used can fall into the categories of Electron microscopy such as transmission electron microscopy (TEM), Field emission scanning electron microscopy (FE-SEM), Environmental scanning electron microscopy (ESEM); light scattering techniques, atomic spectrometry such as inductively coupled plasma mass spectrometry (ICP-MS), single particle ICP-MS and X-ray absorption spectroscopy (XAS); separation techniques such as asymmetric flow-field-flow fractionation, capillary electrophoresis, hydrodynamic chromatography; and electroanalytical techniques such as voltammetry of immobilised particles, particle collision Coulometry. These techniques have been used to measure nanoparticles in consumer products containing or spike with engineered nanoparticles, laboratory release, in vitro digestion simulation, environmental fate, ecotoxicological and toxicological studies for post hoc measurements of in vivo exposure and spiked samples, general analysis of environmental samples. Scenarios considered for such measurements included consumer products, food/biological samples, bioaccumulation, nanoparticle dissolution, release, environmental fate, water and differentiation between naturally occurring and engineered nanoparticles.

Most of these vacuum-based techniques such as TEM, SEM or ESEM are very powerful techniques in providing high-resolution morphology and the chemistry embedded nanoparticles in biological matrix under vacuum and special sample preparations. For example the presence of nanoparticles within a biological matrix can be directly visualised as shown in Figure 5 (Brennan et al. G., SAM, 2017 unpublished data). The TEM specimen was
prepared from HTC116 human colon colorectal carcinoma cells that have been incubated for 24 hours with the gold coated iron oxide magneto-plasmonic nanoparticles. The samples were fixed and centrifuged down into pellet, placed in a resin from which cross sections were cut using an ultramicrotome placed on a TEM grid for imaging. The endosome/liposome collapse shows the nanoparticles uptake into cells, however, direct real life imaging of permeation pathway of nanoparticles into cell still alludes us. Current practices of extracting and isolating nanoparticles from clinical samples do not truly define the clinical significance of detected exogenous nano-sized objects [56].

![Figure 5](image-url)  
**Figure 5.** TEM micrograph of internalised magneto-plasmonic nanoparticles within HTC116 human colon colorectal carcinoma cells [36] Nanoparticles have been internalised into endosomes/liposomes, which have collapsed (lower right).

On the other hand, conventional biochemical assay tests for detecting biological interactions are slow and, in many cases, simply unsuitable due to the requirement of wet-chemical analysis. Furthermore, ambient measurement tools, currently available or being
developed, require customisation to suite the specific needs of a given production environment. For biofunctionalised surfaces, (e.g. those with biomarkers on CMOS chips, enzyme or gene coated stents) front-end characterization is often restricted due to packaging issues and the destructive nature of high-resolution characterization techniques.

There are other areas where metrology can benefit from understanding biological interactions. For example, the nanoarray technology offers overcoming many problems such as large volumes of samples, long incubation times and high limits of detection (i.e. low sensitivity) inherent to current DNA or proteins microarray technologies for gene expression. The extent of detectable non-specific bindings of proteins especially in complex mixtures are generally very small in nanoarrays, the concentrations of which typically lie in the atto-molar (aM) range. The technology to manufacture nanoarrays is well advanced but signal detection from individual elements with adequate contrast and high spatial resolution is difficult. Fluorescence based imaging can provide better detection threshold but their spatial resolutions are limited by diffraction. Scanning probe microscopy offers better spatial resolution with high sensitivity but this technique still require a long scanning time.

Biological systems are intrinsically heterogeneous, comprising distributions of structures, dynamics, kinetic rates, and interactions. Crucial information about these distributions is lost in conventional biochemical and structural methods, which measure the average properties of many molecules. Single-molecule approaches, which reach beyond ensemble-averaged approaches, are therefore particularly powerful and informative, recovering information lost in the ensemble average. Nanoscopy based observation of single proteins, especially membrane proteins, and analyses of their chemical, conformational or folding modification are important to understand the in vivo functions of these proteins. This approach would be particularly powerful to better understand mechanisms such as cell signaling, signal transduction and transport. Electron transfer in cell respiration is coupled
with translocation of protons across mitochondrial or inner bacterial membranes. This primary event of biological energy transduction results in electrochemical proton gradient that is subsequently used in synthesis of ATP. Genetic disorders of the mitochondrial respiratory chain are most commonly characterized by hypotonia, growth retardation, cardiomyopathy, myopathy, neuropathy, organ failure and metabolic derangement. Biochemical investigation of respiratory chain complexes would make a valuable contribution to our understanding of this important class of enzymes. Infrared spectroscopy with a nanoscale resolution would support investigation of single proteins in vivo and allow insights into the mechanisms observed in a number of medical disorders directly related to respiratory chain complexes. For example, the study of the structure and functions of membrane proteins e.g. metalloproteins involved in the respiration in mitochondria can be extended to in situ real time studies.

Vibrational spectroscopies can be used to obtain biochemical fingerprint and the structure of cells and tissues, in a non-invasive and non-destructive manner without requiring fixing or labelling. Hyperspectral mapping of subcellular organelles with features dominated by only a single biochemical component (e.g. cholesterol and phosphatidylcholine) at a nanoscale resolution can be used to generate detailed chemical images revealing the distribution of a number of different cellular components. High spatial resolution would provide a more accurate fingerprinting of biochemical differences between cells and tissues (e.g. tumors). It will also allow investigating more accurately a number of fundamental biological processes, including cell cycle dynamics, cell death, cell differentiation. Finally, in vitro toxicological evaluations of pharmaceuticals, as well as cellular processes within living cells would be made possible with a better subcellular localization.

Imaging the nano-bio and interface can also be important for industrial quality control and predictability in dose delivery of many hybrid devices where molecular therapies are delivered to local diseased sites using state of the art minimally invasive devices and
procedures. It is also critical for the reproducibility and repeatability of bio-electronic devices including biosensors, lab-on-chip devices and electronic noses (in Fig. 4 SPM imprint images and nanoindentation data of a multilayer composite coating for electrowetting applications, are presented). Such methods can also be critical for tracking the biological and environmental fate of nanoparticles especially for the workers who are at the forefront of nanomaterials’ production and faces the highest possible exposure to potentially hazardous type and amounts of nanoparticles. The ability to specify the toxic potential of nanomaterials is a critical component of assigning safety with nanoparticles and would have significant input to the insurability and hence the long term sustainability of nanotechnology industry [57].

Currently available tools and techniques for nanoscale measurements in biological and nonbiological systems can be broadly classified as:

- Vacuum-based techniques,
- Fluorescence Microscopy and its variants,
- X-ray Tomography,
- Magnetic Resonance Imaging and
- Scanning Probe Microscopy and near field approaches.

Table 2 summarizes the strengths and the weaknesses of common microscopic techniques routinely used in materials science and life sciences. X-ray tomography and Magnetic Resonance Imaging have also been used extensively for in vivo imaging. The values for Raman microscopy are theoretical. In practice, Raman microscopy at these levels of resolution is limited by its lower cross-section, which ends up in a very weak signal to merit any imaging. Tip Enhanced Raman Spectroscopy (TERS) has emerged to overcome this limitation.

<table>
<thead>
<tr>
<th>Technique</th>
<th>x-y (nm)</th>
<th>z (nm)</th>
<th>FoV</th>
<th>Label</th>
<th>Finger print</th>
<th>living cells/tissues</th>
<th>3D</th>
<th>Quality control use</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM</td>
<td>~0.5-2</td>
<td>4-7</td>
<td>Wide</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>TEM</td>
<td></td>
<td></td>
<td>Narrow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FM</td>
<td>180-200</td>
<td>450</td>
<td>Wide</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>STED</td>
<td>20</td>
<td>50</td>
<td>Narrow</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
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<td>100</td>
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<td>No</td>
<td>No</td>
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<td>(TEIAS)</td>
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Vacuum based techniques such as electron microscopy and spectroscopy are routinely used for off-line imaging and chemical analysis at nanoscale but due to the requirement of a vacuum, these techniques are typically not suited for studying living cells and artifact-free biomolecules. The methods provide nonetheless very high lateral resolution, down to ~5 nm for Scanning Electron Microscopy (SEM) for example. Chemical information can be obtained by recording Auger electrons, or X-ray emission (energy dispersive X-ray, EDX). Vacuum based surface chemical analyses techniques such as X-ray Photoelectron Spectroscopy (XPS) and Auger Electron Spectroscopy (AES) can also be used for spatial mapping but with varying degree of lateral resolutions. Most commercial imaging XPS (iXPS) equipment offer a lateral resolution of between 1-5 µm, with a 3-10 nm information depth. Scanning Auger electron Microscope (SAM) can achieve very high lateral resolution (30-100 nm) but is limited to conductive samples only. Biological samples releases water to the detriment of the high vacuum needed for such analyses. High pressure (i.e. low vacuum) versions of XPS suffer from poor spectral intensity and resolution and have currently been used with synchrotron sources.

For biological imaging, more common is the use of optical microscopy. Several optical fluorescence based strategies have been developed to allow nanoscale imaging down to a few nanometres. Several breakthrough developments during this period have now established a panel of sub-diffraction imaging strategies [58, 59]). Techniques such as stimulated emission depletion microscopy, STED [60] and stochastic reconstruction microscopy, STORM [61, 62] have been experimentally demonstrated with resolutions of ~20 nm. To achieve spatial resolution below the diffraction limit, and down to a few tens of nanometers or less, fluorescence microscopies rely on the possibility to spatially confine, at a given time, the fluorescence emission to a small volume of chromophores of nanoscale dimensions, sometimes down to single chromophores. The signal remains of course diffraction limited but
the size and location of the fluorescent volume is then either known or recomputable at very high spatial accuracy. For example, to achieve this, in addition to the Gaussian-shaped beam exciting the chromophores within a diffraction limited volume, STED uses a second beam that exhibits a central node. The fluency of this second beam is adjusted to de-excite by stimulated emission these chromophores that are at the edge of the primary irradiated region. It results that only a nanoscale volume of the sample, much smaller than the size of the exciting beam, are left excited and thus capable of emitting the spontaneous fluorescence signal. Areas of biological samples are then made visible with nanometres resolutions through these chromophores.

Nanoscale magnetic resonance imaging (Nano-MRI) has been recently investigated and demonstrated by IBM. Three-dimensional MRI (1H) images of tobacco mosaic virus deposited on a cantilever cooled down to 300 milliKelvin (mK) and scanned within a high-gradient magnetic field were reconstructed with a spatial resolution just below 10 nm. To reduce the thermal noise in the cantilever response samples need to be cooled to milliKelvin temperatures, which makes the whole approach impractical for production lines and most of the laboratories. Nano X-ray computer tomography (nano-CT) has been improved in recent years so that spatial resolutions in three dimensions (< 100 nm) are now possible in laboratories. Currently, the method remains impractical for most applications given the long acquisition times required (i.e., 2 days per measurement). The technique is also not capable of providing clear chemical information on samples. Microscopes based on soft X-ray synchrotron sources allows efficient probing of samples, with a record resolution of ~15 nm using Fresnel zone plates as focusing elements (typical resolution of ~50 nm). Fluorescence from samples can be used to infer chemical information, however in this case at much lower resolution (i.e., several microns). The use of a synchrotron source is not very suitable for commercial applications.
Optical microscopy based on vibrational spectroscopies such as infrared absorption spectroscopy, IRAS, coherent anti-Stoke Raman spectroscopy, CARS [63], sum-frequency generation, SFG [64], and stimulated Raman scattering, SRS [65] involve the generation of images at given spectral wavelengths over an area of interest. These ‘far-field’ spectroscopic techniques analyze the characteristic electromagnetic responses in the vibrational modes that originate from the interactions of materials (organic or inorganic) with the electromagnetic waves used for probing the material. Such characteristic electromagnetic responses reveal dimensional, structural and chemical information without the need for any contrast or stimulating agent (label-free). Imaging is normally achieved by focusing the optical probe beam(s) into the smallest volume possible, and by scanning the sample within this spot in a confocal arrangement. Wide-field imaging, as done in a classical microscope is also possible [64, 65]. These techniques are becoming increasingly popular for imaging samples in life science, materials science and their interfaces. Despite their high throughput imaging the lateral resolution remains moderate due to the diffraction limit, which is roughly one half of the dimension of the wavelength used to probe. For example, in IR microscopy, the best lateral resolution achieved using a synchrotron source is ~2 µm. Most commercial IR microscopes will have a lateral resolution larger than 20 µm; none of them is capable of 3-dimensional analyses. CARS and SRS are advantageous due to the shorter wavelength inherently involved in such techniques and can in principle afford resolutions of a few hundreds of nanometers. Wide-field SFG exhibits a resolution down to a few microns.

The high resolution of SPM has been exploited in obtaining chemical sensitivity using the near field through samples’ autofluorescence, tip-enhanced Raman spectroscopy (TERS) or tip-enhanced IR absorption spectroscopy (TEIAS), which are the SNOM based techniques adapted to probe intrinsic vibrational signatures of samples of both biological and nonbiological origins. These are high resolution, ambient techniques but analysis throughput
is very slow for both routine and industrial applications and must be improved. Multiprobe approaches have been researched as a solution to low throughput but focusing, synchronization and replacement of multiple probes may be cumbersome and time consuming. A revolving cartridge of multiple probes with autofocus and high imaging rate has been proposed that may increase the throughput by reducing time in probe placement, focusing and replacement [66]. SPM probes scan the very surface of samples, which to some extent limits the scope of SPM for three dimensional and interfacial applications. The technique is also limited in providing direct chemical information for which local probe based spectroscopy methods have been developed using the near-optical field created near the probe-material interface.

Tip-enhanced Raman spectroscopy (TERS) benefits from an incredibly large enhancement of the typically very weak Raman emission, by the excitation of local surface plasmons (LSP) at the metal tip apex [64-68]. TERS resolution is routinely of a few tens of nanometre, down to ~15 nm. Because it measures the intrinsic chemical (Raman) signature of samples, it has been developed in numerous laboratories in the last few years and commercial systems are also available. Despite successful demonstration of the technique on several occasions, the low signal throughput usually hampers both imaging and TERS spectroscopy [69]. SNOM approaches that involve probing the sample surface with a tapered optical fibre have also been used for vibrational imaging. Near-field SFG microscopy has been demonstrated with a resolution of ~150 nm [70]. Routine resolution ranges from as low as 100 nm for IR SNOM. In principle, all optical techniques can be (and most have been) adapted to SNOMs.
5. Next generation nanoindentation techniques for fast and high-throughput mapping, rapid prototyping and adhesion assessment on a small scale

Nanomechanical metrology on strongly heterogeneous, multi-phase, composite and nanocomposite materials is a main challenge in both the scientific community and the manufacturing industry [35, 38, 39, 71-74]. As described in the previous chapters, nanoindentation can offer unprecedented mechanical testing performance, in terms of force/displacement resolution and positioning accuracy. In fact, nanoindentation can be used not only for reliable and repeatable measurement of hardness and elastic modulus on a nano-scale (Oliver & Pharr method [5]), but also to investigate mechanical behaviour of very thin films [75], size effects in plasticity of metals [76], failure and crack propagation mechanisms of ceramics [15], residual stresses and fracture toughness [16].

In addition to that, contact mechanics experiments have been demonstrated to be an effective tool for the measurement of adhesive properties of solids on a micro- and nano-scale. In the last few decades, major theoretical efforts have been put into the development of the theoretical and mathematical background for calculation of the Surface Free Energy (SFE) and adhesion from simple load-unload contact mechanics experiments [40, 77-94]. Basing on the original Hertz solutions (1880), where the two solids in contact are assumed to be perfectly elastic and near-contact adhesive forces are neglected, several updated model have been proposed. In the Johnson-Kendall-Roberts model (JKR) [95-97], an additional term related to interfacial adhesion strength is considered in the solution of the contact problem between elastic solids [98]. Because of the adhesive contact, jump-to-contact (or snap-in) and pull-off negative forces are formed during the load-unload cycle. The Derjaguin-Muller-Toporov (DMT) theory represents a further improvement of the JKR model, since it takes into account Van der Waals interactions to be added to the elastic forces, giving rise to additional loads and a more comprehensive description of the adhesive forces. Some examples already
exist in the literature, where either AFM or nanoindentation testing are used to measure the Work of Adhesion from the pull-off force. A more recent investigation [99] has also suggested that the jump-to-contact (or snap-in) force could be used to directly evaluate the Surface-Free-energy of the sample. In this work, a simple energy-based model is proposed for a direct calculation of the Surface Free Energy from direct measurement of the snap-in force and displacement. The latter approach is very convenient in case of testing on nano-patterned surfaces and/or very soft substrates, where plastic deformation or damage during the loading sequence may occur. Despite the large number of theoretical and experimental work in the literature [100-102], there are still major limitations to the effective and efficient applications of such methods to real-time measurement of adhesion in industrially relevant applications, mostly because of:

1. Insufficient control of environmental parameters during the experiments, e.g. humidity, presence of surface contaminations, chemical affinity between the tip and sample, tip wear effects, etc.

2. Limitations of the adopted load-displacement measurement sensors. Since the snap-in and pull-off events are extremely fast events (usually, of the order of 0.1 ms), the time resolution of the displacement sensor must be extremely good to be able to capture with the required accuracy the complete force/displacement profiles. In case of AFM-based techniques, an additional limitation is given by the low compliance of the cantilevers used for testing, which causes unstable snap-in and pull-off events;

Therefore, there is a strong need from research and innovation actions in this field in order to introduce next-generation nanoindentation and AFM procedures for accurate and reliable adhesion testing procedures. Additionally, there is a major requirement from the manufacturing industry to develop fast, in-line and real-time testing procedures for the
assessment of the nanomechanical and adhesive properties in strongly heterogeneous materials, including nanocomposites and nano-patterned surfaces.

This is in full agreement with recent discussions in the scientific community, where high-throughput experimental methodologies have been clearly identified as the KEY for success of the Materials Genome Initiative in USA (or similar actions in Europe, [103]).

In the last few years, a novel high-speed nanoindentation [41, 104] protocol has been proposed in the scientific community, where high-speed contact mechanics experiments were possible with very high displacement acquisition rate. The required performance is obtained by a careful control of the dynamics of the indentation head and a proper correction for the inertial and damping forces that are directly affecting the measurements during a fast indentation process. Using this new nanoindentation protocol, a complete load-unload cycle can be successfully completed in less than one second (1 s) and the full load-unload information is recorded with a frequency of data acquisition of the displacement signal that can reach 100 kHz. In a series of recent papers, revolutionary mapping capabilities have been demonstrated for high-speed nanoindentation testing, where thousands of nanoindentation experiments can be performed in a few hours over large surface areas in heterogeneous materials. Examples include nano-mechanical property mapping in cementitious materials, battery composites and metal matrix composites. By using already established statistical deconvolution techniques [38], robust and reliable information on mechanical property distribution can be achieved for materials with complex phase distribution, including bio-inspired composites and hybrid materials. In the following picture, an example is shown of microstructural vs nano-mechanical mapping on a cement paste after 1 day of hydration. The array of nanoindentation measurements is drawn on the left SEM image. It is worth noting the very good correspondence between the elastic modulus map (where different hydrated and un-hydrated phases are identified) and the SEM (BSE) image. Similar maps can be obtained,
for hardness, displacement into surface and even work of adhesion over a wide range of materials, including composites and hybrids.

Figure 6. High-speed nanoindentation applied to heterogeneous materials: comparison between an SEM image (where the array of performed indentations is also drawn), and elastic modulus high-speed map (obtained from 900 nanoindentation tests that are completed in about 15 minutes) [41]

High-speed nanoindentation can also be used as a next-generation tool for surface nano-patterning over large areas, thus extending the well-established concept of Nano-Imprint Lithography (NIL) [105-113]. The following figures report an example of high-speed nanoindentation lithography (NIHL) on a Bulk Metallic Glass (BMG) sample. A custom-made indenter consisting of an array of truncated cones with sub-100 nm surface features is fabricated by FIB machining [114]. In this specific case, the geometrical features were select with the sole purpose of demonstrating the spatial resolution achievable by FIB milling of a diamond indenter. Different surface features could be milled for creating custom-made geometries by this technique. Finally, the indenter is used to pattern a large area substrate by realizing an array of indentations at a prescribed load, using the high-speed modality, as reported in Figure 6. The new capabilities given by NIHL could be extremely useful to develop prototype pattern surfaces, where novel nano-scale architectures could be rapidly assessed as potential candidates for upscaling, as a direct support of other industrial processes.
(e.g. micro-contact printing lithography). Moreover, the use of a depth-controlled fast imprinting technique could be important for fine-tuning and optimization of punch geometry as a function of the specific material’s behaviour.

In the example reported below, an array of holes (with sub-100 nm features in their internal surface) is fabricated over a large area (> 1 mm$^2$) in a few hours. A statistically relevant change of water contact angle was achieved after patterning, even though the specific design for this surface architecture was not focused on adhesion.

**Figure 7.** Nanoindentation high-speed lithography (NIHL). (a-b) FIB fabrication of diamond flat punches with sub-100 nm features; (c-d) nanoindentation lithography over large areas with sub-100 nm resolution obtained by NIHL; (e-f) changes

The possibility for high-frequency (100 kHz) displacement data acquisition also opens the way for unprecedented adhesion experiments to investigate surface free energy modifications on a nano-scale in advanced nanostructured materials and/or architecture surfaces (Figure 7,
The following picture reports a typical load-unload curve obtained by high-frequency nanoindentation, where the snap-in and pull-off adhesion events are highlighted. By using 100 kHz data acquisition frequency, the snap-in and pull-off adhesion events are fully captured and can be effectively used for work of adhesion and surface free energy calculations. The example in Figure 7 (d) reports that remarkable differences for snap-in forces can be achieved in case of surface patterned PMMA samples, with respect to the values obtained for the reference (smooth) surface. Additionally, the same figure shows that the interaction profile is non-linear. This information can be extremely valuable for further refinement of the models adopted for surface free energy calculations.

**Figure 8.** High-frequency data acquisition nanoindentation applied to surface free energy measurements on homogeneous and nano-patterned PMMA samples. (a) full load displacement curve, showing the snap-in and pull-off events; (b) detail of the snap-in load-displacement event; (c) 100 kHz displacement vs time data acquisition; (d) examples of snap-in event on reference PMMA and patterned PMMA surfaces, showing a difference in the snap-in forces.
It is worth noting that analysing the snap-in event to calculate SFE is extremely convenient for the quantitative analysis of adhesion phenomena on very small areas. Possible areas of applications are the analysis of SFE modifications induced by nano-patterning procedures and studies on cell adhesion mechanisms on a very fine scale. Additionally, the technique is very fast and non-destructive, thus allowing for high throughput reliable characterisation, including in-line and real-time testing. In Figure 8, load versus displacement is presented, as the tip approaches the surface, it snaps into contact because of surface forces in the surface of polydimethylsiloxane (PDMS) (with schematic of mechanism as inset). Shaded area is reported as Work of Adhesion and can be correlated with hydrophilicity/ hydrophobicity of the surface, with a use of a proper tip [99, 115].

Figure 9. Load versus displacement as the tip approaches the surface, snaps into contact because of surface forces in the surface of PDMS [37]
6. Challenges and new approaches towards imaging nano-bio interactions and interfaces

Nanometrological challenges should be addressed to successfully develop the nanomechanical instrumentation needed to support the future nanotechnology industry. To meet these, joint efforts should focus on the development of standards and calibration methods, accurate predictive modelling tools, and reliable, fast, multifunctional, quantitative instrumentation techniques. A key approach will be to develop those methodologies with the greatest potential in order to achieve breakthroughs and enable successful commercialization of nanotechnology.

Instrument development is then needed to foster the progress of nanotechnology by bridging the existing metrological gaps. Measurement capabilities should involve:

- Advanced sensors/actuators with linear response, resulting in enhanced spatial and lateral resolution.
- Increased speed of characterization and sample preparation (finishing) leading to productivity improvements.
- Performance of in situ nanomechanical measurements under a wide range of challenging working conditions (temperature, aqueous environment etc.).
- Control of thermal expansion of transducer aiming at reducing the thermal drift phenomena.
- Development of robust indenter tips appropriate for atomic-scale nanomechanical testing.

Calibration standards materials and methods are still needed in mechanical nanometrology, whilst manufacturers of existing instrumentation provide their own calibration standards. Within this area many challenges are met, such as:
- Development of consensus standards and officially certified by international organizations for routine verification of machine performance and compliance.
- Establishment of traceable and universal standards.
- Reliable force and displacement calibration to perform measurements with piconewton and picometer resolution.
- Development of methods for producing highly precise and well-characterized indenter tip geometries.

Standardisation and metrology go hand in hand for successful commercialisation of industrial products, processes and technology. Inspection, monitoring and quality assurance are critical requirements for the success of manufacturing: measurement. Metrology, the science and act of measuring, is required for technological, market and consumer confidence underpinned by standardisation. Metrology primarily assures whether object manufactured is actually suitable for the purpose intended in both functionality along with its tolerances. It is also important to control and optimize a manufacturing process towards achieving product and performance standards through appropriate metrology. This is extremely important for emerging manufacturing and product portfolios emanating from Europe’s outstanding scientific and innovation value chain. While we can make some very complex and unconventional shapes and structures using, for example nanotechnology, biomimetics or additive manufacturing we are yet to put in place a robust quality assurance scheme and standardisation mainly due to lack of appropriate and enough metrological tools and methodologies. The success in setting appropriate metrology tools, techniques and methodologies in these types of products and processes is required to enable a high confidence in product, improve energy efficiency and reduce scrap material, redundant processing time, cost and environment. This can be achieved through validation of measurement procedures, new standard reference materials,
metrological traceability, cross-verification through multimodal round robin testing in multiple locations.

Metrology can be on-line, in line or off-line. Europe has a leading edge in the field of metrology instrumentation and products. The field of metrological tools and methodologies however face a huge challenge with a drive towards higher value added, lower cost, complex manufacturing e.g. those foreseen in “Manufacturing 4.0” initiative, or nanotechnology.

Current optical, mechanical, magnetic and electrical metrological tools are not adequate to enable fast and reliable on and in line metrology in a cost effective way as it may be required for additive manufacturing. Only a few techniques exist that can measure and standardize physico-chemical properties of nanoscale materials at low dimensional measurements within a production process. For example, methods for the determination of particle size should be linked with standardized measurement techniques. Measuring texture and in-process defects largely depends on destructive and non-destructive off-line characterization techniques. This roadmap highlights the key actions required for enabling the drive towards real time in line quality assurance along with the formulation of legal-regulatory guidelines for proper standardisation.

Measuring nano-bio interactions and their interfaces has been considered as ‘the most difficult challenge to the nanometrologist’ [116]. The surface of nanoparticles that has been characterised before the introduction to a biological milieu is most likely to have been altered due to adhesion of biomolecules in the pathway. These unintentional but unavoidable biofouling will have important repercussion on the surface properties of the nanoparticles and in the way these particles interact. Any foreign object that enters the sterile body system is subject to an immune response. While it is mostly seen as a nuance due to non-specific adhesion of proteins at the surface of the foreign objects it is also possible to custom-design nanoparticles surfaces to overcome non-specific adsorption, promote specific adsorption and
enhance bioavailability and biodistribution [117-119]. Predictive relationships between structure and activity of nanoparticles, on the other hand, can be inferred from a better understanding of the nano-bio-interface [120].

As discussed in the preceding section, a number of common imaging/measurement techniques are simply not suitable to measure nanoscale dimensions and structures of biological species without special sample preparation, special conditions during measurements or causing damage. Specimens where biological species coexist with e.g. an inorganic nanomaterial are even more cumbersome. For example, high vacuum electron microscopy techniques can image the inorganic nanomaterial but the high excitation voltage used for such imaging can simply burn the biological/polymeric part of the specimen. Coming from the other end, the inherent permeable nature of biological species made them ideal for various ‘staining’ where luminescent or fluorescent probes or ‘labels’ are used for contrast enhancement. Very often, the dimensions of these probes are within the nanoscale, so their size and chemical effect may become influential while imaging them at high resolution. The requirement of high vacuum in electron microscopy, despite its high lateral resolution, has limited the information that this technique can provide for biology, where water plays a vital role. A number of such limitations are overcome in low temperature electron microscopy such as cryogenic transmission electron microscopy (cryo-TEM) or high pressure scanning electron microscopy such as Environmental scanning electron microscopy (E-SEM) but there is always a trade-off: high investment cost, complicated and specialized sample preparation, or a compromise in the lateral resolution. It is no surprise that currently, quality assurance and performance guarantee of nano-bio products largely depends on either offline/offsite high lateral resolution techniques, or on/in line low lateral resolution metrological tools.

Dimensional resolutions that can be obtained from super-resolution optical nanoscopy (STED and related techniques) are limited to the size and dispersion of the chromophores and thus
offer very high spatial and axial resolution. The use of chromophores may be problematic in many cases especially for inorganic nanostructures and their interface with biological species as the former may not be very permeable to allow such chromophores thus making super-resolution technique counterproductive. Chromophores are also subject to degradation and may be toxic. Most importantly, these super-resolution techniques are not chemically sensitive. Chemical imaging is important as the chemical constituents and bonding are fundamental to the construction of the building blocks, structure, nanostructure and microstructure of both biological and non-biological species.

Currently, optical spectro-microscopy techniques are not capable of competing with the spatial resolution affordable with near-field scanning optical microscopies. Chemical imaging with optical spectro-microscopy techniques in the far field is limited by diffraction limit whereas in the near field they suffer from low throughput, narrow field of view and very shallow axial resolution. It seems thus that a breakthrough in chemical imaging would be achieved if one could realize a far-field vibrational microscopy with sub-diffraction resolution. Analyzing the fundamental principles behind the extraordinary resolution of these fluorescence microscopies may allow new concepts to develop high-resolution vibrational spectro-microscopies [121, 122].

CARS and SFG are two complementary optical non-linear vibrational spectroscopies that are exploited in material sciences, physical chemistry, as well as in life sciences. CARS is commonly used for three dimensional imaging (e.g. of biological samples), sometimes in conjunction with SFG. However, SFG is better suited for the analysis of thin films and interfaces e.g. in nano/bio sensors, biofilms and molecular electronics due to the exclusive sensitivity to non-centrosymmetric media so common in biological species. SFG relies on two synchronous, generally picosecond long, laser pulses of infrared (IR) and visible wavelengths that are superimposed on the sample. The second order polarization induced at the interface
leads to the emission of photons whose energy corresponds to the sum of the energies of the visible and IR beams. To establish a vibrational spectrum, the IR wavelength is scanned and SFG resonances are seen when its wavelength matches with vibrational quanta of sample. The spectra are thus reminiscent to IRAS albeit with different selection rules. However, the SFG wavelength is in the visible range and the signal is thus readily detectable with a standard photomultiplier or a cooled charge-coupled device (CCD) camera.

Two concepts for sub-diffraction CARS imaging have been proposed. They are based on using a control beam akin to STED, but in this case for either breaking the Raman coherence or entertaining Rabi oscillations [62, 123]. We are however not aware of any experimental evidence supporting these concepts. Similarly, sub-diffraction SFG imaging can be envisaged if one can identify a mechanism for locally quenching the SFG susceptibility using a specially profiled control beam. For SFG and, as a matter of fact, also for IRAS, a similar mechanism exists and has been experimentally exploited in the past to measure the relaxation time of targeted vibrational modes. The method is based on a pump-and-probe strategy, and involves saturating the vibration with an IR pump beam before SFG (or IRAS) probing [124-126]. The signal is quenched for pump-probe delays shorter than the vibration lifetime, and recovered for longer ones. Experimentally, one needs to ensure that the IR pump and the visible and IR probe pulse widths are shorter or of the order of magnitude of the lifetime involved, which are typically around 10 ps for adsorbents on metal substrates. Nanoscopes can be constructed using such super resolution approach in the Infrared [121, 122] and is currently under construction in the authors’ group.

New techniques such as super-resolution Infrared nanoscopy and CARS microscopy will have the advantage of using the far field construction for combining both high lateral resolution with moderate to high throughput. Far-field approaches are on the other hand compatible with the measurement of thicker or buried samples and with three-dimensional imaging. These are
fundamental qualities for probing many of the biologically relevant samples. They also completely alleviate the experimental complexity in handling the tip in SNOM, and afford larger fields-of-view. Ideally, one would want to combine the resolution achieved by near-field instruments with the advantages of far-field spectro-microscopies.

Another approach in measuring nano-bio interface is to use a hybrid approach. For example, combining high resolution of an SEM with an ambient Raman spectro-microscopy technique, or combining TERS, micro-Raman and aperture based SNOM in an integrated table-top SPM. This approach has the benefit of bringing the best from different techniques in analysing the sample within the same field of view. Multimodal imaging has been very popular in conventional SPM especially in electrical force probing where the same probe can reveal topography and different electrical force functional within the same field of view [127]. Example includes electrostatic force microscopy, magnetic force microscopy and piezoresponse force microscopy. An extension of this to non-SPM imaging by integrating complementary or even contrasting microscopy techniques can bring tremendous benefits while eliminating the need for transporting samples from one microscope to another and struggling to find in the latter the same area analysed in the former. Integrating two (or more) microscopes into one has been predominantly a realm of the physicists but has recently gained momentum among non-physicists as the benefits of such hybrid systems are becoming clearer [128]. Fortunately, a number of SPM manufacturers such as Bruker, JPK and NT-MDT are offering such hybrid systems as commercial products and many more of such microscopes are to come in near future for imaging of nano-bio interactions and interfaces.

Among other non-image based techniques, gravimetric methods such as quartz crystal microbalance have been very sensitive and widely popular in investigating protein interactions. These techniques are typically incapable of providing direct dimensional or chemical information that are generally required in nano-bio interactions such as size,
morphology and chemistry. Electrical and electronic measurements for quantitative measurements of biological system are in its early days but offers a huge potential if chemical/biochemical selectivity can be achieved. Techniques such as electronic DNA sequencing, protein analysis and single molecule mass spectrometry have the potential to revolutionize nanobiometry when correctly validated by other metrological tools. However, for now, imaging remains at the core of the challenges of measuring nano-bio and their interactions and will need to be addressed first to support the huge scientific and commercial potential that the field offers.

There is no doubt that the burgeoning nanomedicine industry and the field of occupational health in the nanotechnology industry can greatly benefit from these developments. The translation of these tools and methodologies to industrial production sites is however a big challenge especially when one considers that high resolution imaging techniques including electron microscopy have not proceeded beyond the level of off-line metrology even in most of the advanced semiconductor manufacturing. Further research effort must be endeavoured in this space. Two critical steps must be adopted to ensure the translation of these new laboratory based instrumentation to industrial metrology:

(a) firstly a joint research/coordination effort between instrumentation physicists, application scientists (chemists, molecular biologists, materials scientists and physicists), metrological tool and component developers, original equipment manufacturers (OEMs), metrological instrumentation companies and academic and industrial end users;

and (b) secondly, a phase approach so that industry can benefit for now from moderate resolution but high throughput techniques while waiting for high throughput, high resolution techniques are being developed and customized to targeted industrial needs.
7. From laboratory to industrial nanometrology: implications for design

Fast and reliable nano-characterisation tools are needed to speed up and make more effective the design and introduction into the market of nanostructured materials and micro/nano-devices. A reduction of designing and production costs can be associated to the development and validation of fast and in-line nano-characterisation methods.

To achieve such goal, experimental studies should lead the technological background in parallel to design and manufacturing practices, in order to improve the accessibility of nanomechanical instrumentation and metrology to industrial applications (evolution of mechanical nanometrology is depicted in Figure 9).

Therefore, a main breakthrough into nanomaterial design can be associated to the development of the fast, in-line and real-time metrological characterisation methods that are described in this article.

First, the main technological advancement is associated to the increase of data acquisition speed and frequency, the development of fast and automated procedures for data quality and risk assessment. Rapid characterisation methodologies are less sensitive to environmental noise and can open the way to the introduction of nano-scale characterisation methods inside the production line (in-line and real-time characterisation), thus making more effective the design and optimisation processes and reduce time-to market of advanced products.

Several additional aspects can be directly associated to the exploitation and transfer of nano-scale mechanical characterisation tools to industrial processes, with some direct implications for design:

- The establishment of industrial best practice (methodologies) and standard operating protocols for end-users, thus increasing speed of development in industries;
• The *digitisation* of materials supported by advanced characterisation and establishment of novel concepts for material property metadata to support materials modelling;
• Implementation of workflows into a digital form, making industrial processes more transparent;

![Diagram](image.png)

**Figure 10.** The evolution of mechanical nanometrology towards industry.

There are other areas where metrology can benefit from understanding biological interactions. For example, the nanoarray technology offers overcoming many problems such as large volumes of samples, long incubation times and high limits of detection (i.e. low sensitivity) inherent to current DNA or proteins microarray technologies for gene expression. The extent of detectable non-specific bindings of proteins especially in complex mixtures are
generally very small in nanoarrays, the concentrations of which typically lie in the atto-molar (aM) range. The technology to manufacture nanoarrays is well advanced but signal detection from individual elements with adequate contrast and high spatial resolution is difficult. Fluorescence based imaging can provide better detection threshold but their spatial resolutions are limited by diffraction. Scanning probe microscopy offers better spatial resolution with high sensitivity but this technique still require a long scanning time.

Biological systems are intrinsically heterogeneous, comprising distributions of structures, dynamics, kinetic rates, and interactions. Crucial information about these distributions is lost in conventional biochemical and structural methods, which measure the average properties of many molecules. Single-molecule approaches, which reach beyond ensemble-averaged approaches, are therefore particularly powerful and informative, recovering information lost in the ensemble average. Nanoscopy based observation of single proteins, especially membrane proteins, and analyses of their chemical, conformational or folding modification are important to understand the in vivo functions of these proteins. This approach would be particularly powerful to better understand mechanisms such as cell signalling, signal transduction and transport. Electron transfer in cell respiration is coupled with translocation of protons across mitochondrial or inner bacterial membranes. This primary event of biological energy transduction results in electrochemical proton gradient which is subsequently used in synthesis of ATP. Genetic disorders of the mitochondrial respiratory chain are most commonly characterized by hypotonia, growth retardation, cardiomyopathy, myopathy, neuropathy, organ failure and metabolic derangement. Biochemical investigation of respiratory chain complexes would make a valuable contribution to our understanding of this important class of enzymes. Infra-red spectroscopy with a nanoscale resolution would support investigation of single proteins in vivo and allow insights into the mechanisms observed in a number of medical disorders directly related to respiratory chain complexes. For
example, the study of the structure and functions of membrane proteins e.g. metalloproteins involved in the respiration in mitochondria can be extended to *in situ* real time studies.

Vibrational spectroscopies can be used to obtain biochemical fingerprint and the structure of cells and tissues, in a non-invasive and non-destructive manner without requiring fixing or labeling. Hyperspectral mapping of subcellular organelles with features dominated by only a single biochemical component (e.g. cholesterol and phosphatidylcholine) at a nanoscale resolution can be used to generate detailed chemical images revealing the distribution of a number of different cellular components. High spatial resolution would provide a more accurate fingerprinting of biochemical differences between cells and tissues (e.g. tumors). It will also allow investigating more accurately a number of fundamental biological processes, including cell cycle dynamics, cell death, cell differentiation. Finally, *in vitro* toxicological evaluations of pharmaceuticals, as well as cellular processes within living cells would be made possible with a better subcellular localization.

In order to reach these goals and thus reach a low cost production, a closer cooperation between industrial sectors, manufacturers of measurement instrumentation, academic research community, national metrology institutes (e.g. NMIs) and standards organizations (e.g. ISO, CEN) is necessary. The intention is to introduce industrial mechanical nanometrology into production processes, to meet the industry goals when cost reduction, work-piece quality improvement and process stability safeguard will be accomplished. The increased speed of sample preparation and characterization, the upscale feasibility, the amelioration of spatial and lateral resolution towards quality assurance, the development of multifunctional, reliable techniques and finally, the improvement of automation in measurement process will encourage the introduction of nanomaterials into the world market (Fig. 10). This closer cooperation has to focus on the following points:

- Measurement Instrumentation
- Calibration and Calibration Standards
- Tolerances and Tolerability at the Nanometer Scale
- Parameters and Measurands in NanoMetrology
- International Standards for NanoMetrology

![SEM micrograph showing a 3D printed structure: evaluation of layers cohesion, towards quality control.](image)

**Figure 11.** SEM micrograph showing a 3D printed structure: evaluation of layers cohesion, towards quality control.

3. **Concluding remarks**

Nanometry is a multidisciplinary field and covers a wide range of measurement methods, includes technical goals and visions that have been primarily driven by the need to render more efficient, economic and less environmentally sensitive manufacturing processes, while at the same time achieve the optimization of materials performance and efficiency of the characterization techniques. It refers to measurement techniques that assess properties of materials at the nanoscale level. Within this context, we have discussed current and emerging techniques available for characterization of mechanical and nano-bio interface properties for nanometry and elaborated the need and means to reduce current laboratory based
characterization into industrial metrology practices as the scope of nanometrology extends far beyond off-site, laboratory-based measurements of dimensions only.

We have also highlighted the need for multifunctional instrumentation, traceable and standardized methods, and modelling and simulation tools for unambiguous interpretation of characterization data for nanotechnology-based products. The evaluation of nanomechanical properties, determination of stress-strain, time-dependent behaviour, surface/interface and viscous response of materials (metals, polymers, ceramics, hybrid, composites and soft matter) especially in embedded environments including e.g. biological media have been discussed within the purview of manufacturing processes, standardization and optimization. Finally, we have discussed the potential implications for design and the scientific and technological challenges that are to be addressed to achieve fast, in-line, real-time, reliable, repeatable and predictive metrology to underpin upscaling nanomaterials and nano-enabled products from the research field to industry and market.

More specifically, in the field of mechanical nanometrology, future nanomechanics assessment will require techniques capable of performing rapid, accurate, predictive and well understood results. The combination of experimental abilities together with theoretical/computational capabilities is key aspect to understand and predict the mechanical behaviour of matter at the atomic level. The use of vacuum-based electron microscopy, which has been extremely successful in bringing high-level innovation in the field of inorganic nanomaterials and related technology, is problematic when it comes to the imaging of nanomechanical properties and nano-bio interactions. A number of new techniques that overcomes the problems of vacuum, continuous high-energy beams and other limitations of electron microscopy have been discussed. Newer approaches such as super-resolution far field spectro-microscopy (e.g. those using vibrational spectroscopy) or hybrid nanoscopy are on the horizon and can provide exciting opportunities in the field of nano-bio interactions and
interfaces in near future. A translation of these techniques to study nanomechanics and nanobio interactions and interfaces in an industrial scenario, while highly desirable, will need time and planned intervention. The dissemination of outputs from the use of such metrological tools to industrial stakeholders and general public will promote the upscale and commercialization of nano-based products.

Reference list


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Graphical abstract
Highlights

Nanometrology tools for Nano-Manufacturing and Nano-Bio Interface are reviewed

High-speed nanoindentation as a novel tool for property mapping and nanopatterning

AFM Imaging nanobio interface can become a cornerstone in the metrology of bio-nanotechnology

Improvement of nano-scale characterisation can have Impact for Design

Real-time nano-characterisation is the key for upscaling nano-enabled products to market