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RESEARCH ARTICLE

Label-free super-resolution imaging below 90-nm using photon-reassignment

[version 1; peer review: 3 approved with reservations]

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Abstract

Background: Achieving resolutions below 100 nm is key for many fields, including biology and nanomaterial characterization. Although nearfield and electron microscopy are the gold standards for studying the nanoscale, optical microscopy has seen its resolution drastically improve in the last decades. So-called super-resolution microscopy is generally based on fluorescence photophysics and requires modification of the sample at least by adding fluorescent tags, an inevitably invasive step. Therefore, it remains very challenging and rewarding to achieve optical resolutions beyond the diffraction limit in label-free samples.
Methods: Here, we present a breakthrough to unlock label-free 3D super-resolution imaging of any object including living biological samples. It is based on optical photon-reassignment in confocal reflectance imaging mode.
Results: We demonstrate that we surpass the resolution of all fluorescence-based confocal systems by a factor ~1.5. We have obtained images with a 3D (x,y,z) optical resolution of (86x86x248) nm^3 using a visible wavelength (445 nm) and a regular microscope objective (NA=1.3). The results are presented on nanoparticles as well as on (living) biological samples.
Conclusions: This cost-effective approach double the resolution of reflectance confocal microscope with minimal modifications. It is therefore compatible with any microscope and sample, works in real-time, and does not require any signal processing.

Keywords

Super-resolution imaging, Label-free imaging, confocal reflectance microscopy

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Plain language summary

The resolution of an imaging system is a key parameter since it allows observation of smaller details and thus increases the knowledge about the observed sample. Although essential for studying living samples, optical microscopy has intrinsic resolution barriers which preclude the observation of details smaller than a few 100's of nanometer without modifying the samples (e.g. by adding fluorescent tags). However, reaching the nanoscale on unmodified objects without damaging them is essential to understand the intimate structure of the matter. Here we propose a method based on point scanning (confocal) microscopy that allows resolution bellow 90 nm on any samples and we demonstrate our imaging capability on living cells and nanomaterials.

Introduction

Achieving resolutions below 100 nm is key for many fields, including biology and nanomaterial characterization. Although nearfield and electron microscopy are the gold standards for studying the nanoscale, optical microscopy has seen its resolution drastically improve in the last decades[1–6]. So-called super-resolution microscopy is generally based on fluorescence photophysics[7] and requires modification of the sample at least by adding fluorescent tags, an inevitably invasive step. Optical imaging systems based on confocal microscopy (CM) grant both efficient 3D resolution and optical sectioning ability, and are thus widely spread. The majority of CMs are dedicated to image fluorescent-labeled samples, providing images with molecular specificity. The last three decades of improvement are therefore chiefly linked to the enhancement of fluorescence imaging[8–10]. However, fluorescent tags are dim and fragile, which limits both the acquisition rate and the duration of studies. Labeling is moreover invasive by nature, and it remains very challenging and practically impossible to label samples while ensuring reliable innocuousness (e.g. human grafts or embryos). Label-free imaging is thus key for many applications, including medical diagnostics, and is generally based on recording the light back-scattered by the sample. In this scope, optical coherence tomography[11–13] - including its confocal scheme[14]- and reflectance confocal microscopy (RCM)[15] are the most used techniques. RCM has been demonstrated to be efficient for various biomedical applications including skin imaging[16–18], ophthalmology[19,20], and neuro-biology[21,22]. However, label-free microscopy remains limited in term of resolution as compared to fluorescence-based systems.

One elegant and efficient way to increase the resolution of CM-based setups consists not only of counting the number of photons passing through the confocal detection pinhole with a mono-detector (e.g. (avalanche) photodiode) but effectively recording the spatial distribution of the light transmitted by the pinhole using a 2D sensor (e.g. CCD or CMOS camera). This 2D signal is then (digitally or optically) shrunk before shifting to the next scan point in the sample. Optical super-resolution is obtained since the collected point spread function (PSF) is re-allocated into a distance where the spatial information of the adjacent PSFs can be optically resolved. The concept of photon reassignment has been originally described by Sheppard[23] and has been implemented in fluorescence CM[24–26] (resolution gain of about x1.5), Raman microscopy[27] (resolution gain up to x1.41), and two-photon microscopy[28,29] (resolution gain x1.81). However, by applying this concept to RCM, it is possible to obtain a true doubling of the lateral resolution since there is no Stokes-shift between the excitation and detection wavelengths. DuBose *et al.* recently demonstrated the first example of photon reassignment for resolution enhancement in reflectance ophthalmology[30] (i.e. with micrometer resolution, the eye being the last focusing optics).

We present in this article a method to enhance CM resolution by using a fully-optical photon reassignment method and a reflectance-based imaging scheme. This approach grants: (i) instant super-resolution imaging even at depth (> 100 µm) without any computation; (ii) a lateral resolution below 90 nm, an axial resolution below 250 nm using visible light; and (iii) compatibility with any sample including nanomaterials and living label-free biological samples.

Results

We have designed an approach with a penetration depth of hundreds of micrometers, low photo-induced damage (i.e. no-UV light, average light power over the field of view < 100 W/cm^2 for a signal to noise ratio > 100), a temporal resolution below 1 second, and a spatial resolution better than 90 nm. To do so, we use a 445 nm light source and a high numerical aperture microscope objective compatible with thick sample imaging (Silicon immersion 60x, NA=1.3, WD=0.3mm) (see Figure 1a, Methods and associated Figure 5 for a complete description of the optical setup). The laser is focused on the sample with a diffraction-limited size and laterally scanned with a 2D-mirror. The back-scattered light is then filtered by a confocal pinhole. The image of the confocal pinhole is re-scanned directly onto a 2D camera sensor with a doubled scanning-amplitude as compared to the object plane. This induces a two-fold reduction of the effective PSF in the image plane. The diffraction-limited PSF of diameter d is collected from the sample with a separation distance of *s*, as illustrated in Figure 1a.

The collected PSF is rescanned into the 2D detector, maintaining the same diameter d but with a separation distance of 2*s*. This fully-optical photon-reassignment directly leads to super-resolved images without requiring any computation (see *Extended data: Annex 1* for theoretical explanations)[31]. We named this approach rescanned-RCM as compared to regular-RCM, where the image is reconstructed point-by-point in a de-scanned mode. As an example, in Figure 1b-f the resolving capabilities of rescanned-RCM is largely improved as compared to regular-RCM when imaging nanoparticles (100-nm gold nanobeads). In Figure 2, an example of resolution enhancement on high-resolution US Air Force resolution target is also presented.

To quantify the performance of our setup, we focus on (i) the lateral and axial resolution enhancement, we then discuss

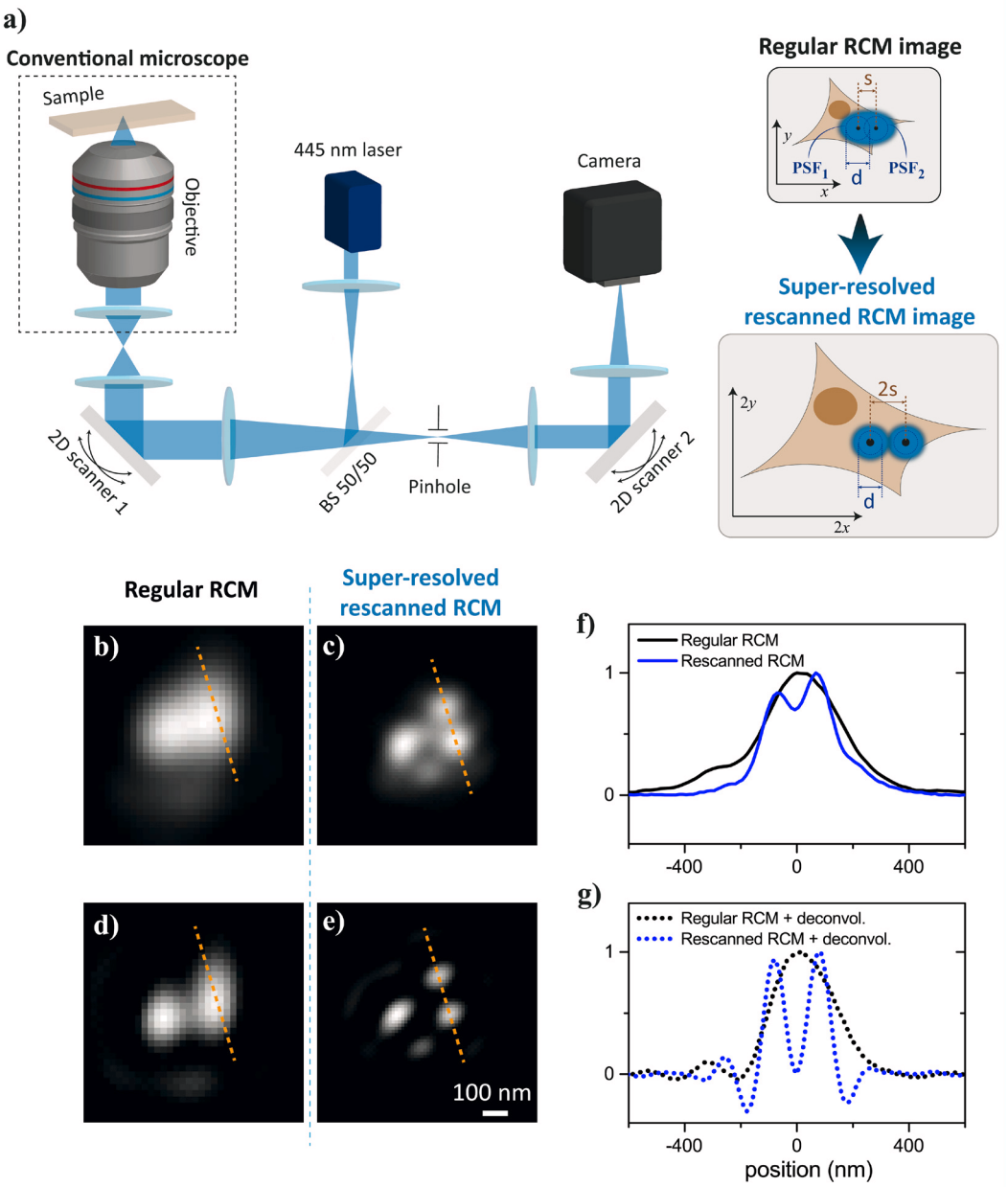


Figure 1. (**a**) Experimental setup and concept to achieve label-free confocal super-resolution, BS 50/50 is a 50%;50% non-polarizing beamsplitter (see Methods for detailed information). (**b**) 100 nm gold beads observed with conventional reflectance confocal microscopy (RCM). (**c**) Same zone as for ***(b)*** observed with rescanned-RCM. (**c**) Deconvolution of ***(b)***. (**e**) Deconvolution of ***(c)***. (f) Line-out of ***(b,c)***. (**f**) Line-out of ***(d,e)***.

(ii) the background rejection capability and (iii) present 3D biological sample reconstructions on quasi-metrological samples (diatom shells in water), and living adherent cells with a focus on intra-cellular organelle dynamics.

Resolution enhancement

Here we compare the lateral and axial 3D PSF and the associated 2D and 3D modulation transfer function (MTF) of both regular-RCM and rescanned-RCM on 60 nm gold nanoparticles (see Methods) acting as point-objects. For a fair comparison between an optimal conventional RCM (recorded on a monodetector) and the super-resolved rescanned-RCM, the optical setup has been upgraded to have both RCM modalities running in parallel for simultaneous acquisition (see Methods for the detailed dual-modality setup). Qualitatively first, the PSF size in the rescanned mode is reduced in comparison with

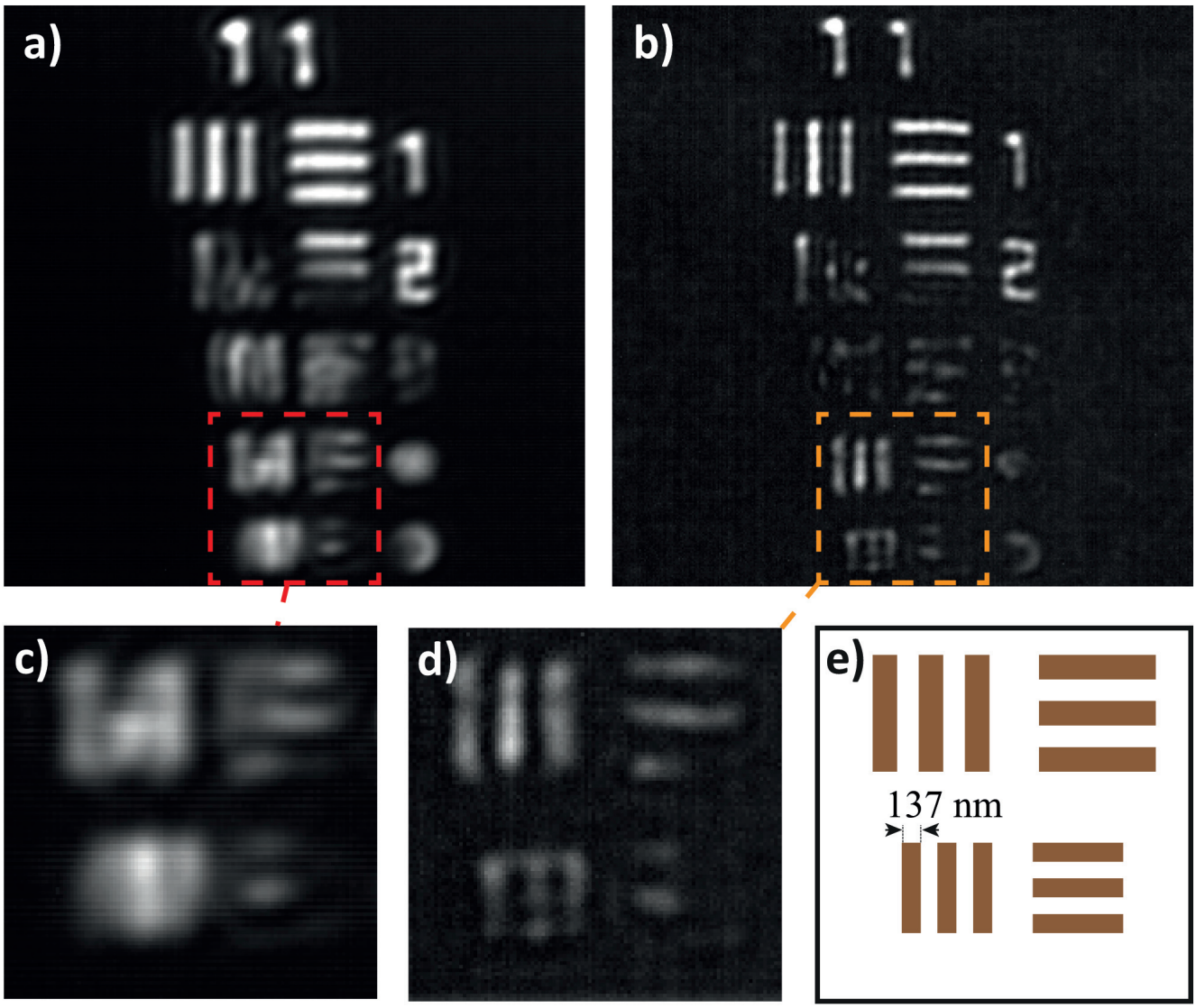


Figure 2. High-resolution positive US Air Force target element 11 observed by (**a**) regular reflectance confocal microscopy (RCM); (**b**) re-scanned RCM; (**c**,**d**) zoom in of the elements 11-4 and 11-5 from the target in ***(a)*** and ***(b)***, respectively; and (**e**) scheme of the theoretical resolution target. Images are 2D deconvolved.

the standard PSF of a regular-RCM (Figure 3a–d). The gain is even clearer when looking at the Fourier domain: the frequency support of the rescanned-RCM image is extended as compared to the frequency support of the regular-RCM image (Figure 3e and 3f).

To quantitatively determine the actual resolution of the microscope and its imaging performance, we have computed the 2D MTFs from both re-scanned and regular RCM frequency supports (Figure 3g). The lateral resolutions of the microscopes have been measured to be $r_{xy}^{RCM} = 171 \pm 5$ nm for the regular RCM and $r_{xy}^{srRCM} = 86 \pm 3$ nm for the re-scanned RCM (consistent with the theoretical resolution value $r_{xy}^{th;srRCM} = \frac{\lambda}{4NA} = 86 \pm 5$ nm). A twofold lateral resolution enhancement as compared to the regular RCM can thus be achieved using the setup in Figure 1.

Although the effect on the axial resolution of re-scanning has been investigated for florescence CM (and even enhanced using structured illumination[32]), we didn't find any demonstration of the effect on the axial resolution for the reflectance-imaging scheme. Figure 3b and 3d demonstrate that rescanning the pinhole-image on a 2D sensor not only increases the lateral resolution but also the axial one. The

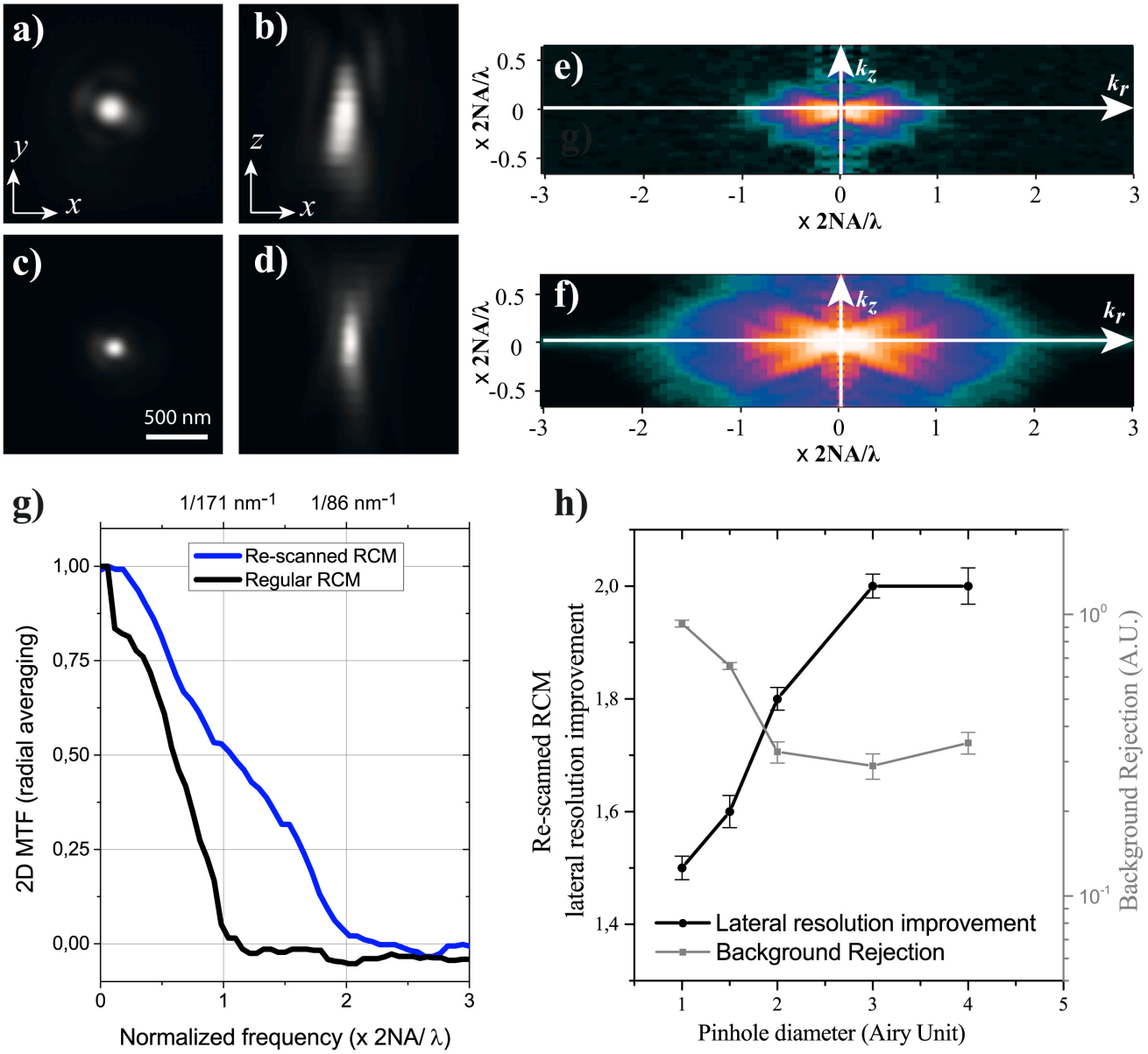


Figure 3. Experimental results of the point spread function (PSF), modulation transfer function (MTF), and background rejection of regular (pinhole of 1 Airy-size) and re-scanned super-resolved reflectance confocal microscopy (RCM), measured on 60 nm gold beads embedded in immersion oil. (**a**) (x, y) PSF of regular RCM; (**b**) (x, z) PSF of regular RCM; (**c**) (x, y) PSF of rescanned-RCM; (**d**) (x, z) PSF of rescanned-RCM; (**e**) numerical 3D Fourier transform of regular RCM PSF (radial average of each k_z plane); (f) numerical 3D Fourier transform of rescanned RCM PSF (radial average of each k_z plane); (**g**) 2D MTF of regular and re-scanned RCM obtained by radial average of ***(a)*** and ***(c)***, respectively; (**h**) experimental results on the relation between the re-scan pinhole size and both lateral resolution and background rejection in the microscope. Resolution and background rejection normalized by those measured with regular RCM (pinhole size of 1 Airy Unit).

frequency support (Figure 3e and 3f) is also increased along the axial direction k_z when using re-scanned RCM. We experimentally determined the axial resolution of the re-scanned RCM to be $r_z^{srRCM} = 248 \pm 8$ nm as compared to the regular RCM theoretical axial resolution of $r_z^{th;RCM} \approx \frac{1.4\lambda}{NA^2} = 373$ nm. This gain of 1.5 ± 0.05 is consistent with the experimental gain measured at 1.4 ± 0.1.

Relation between background rejection and resolution

Another important aim of confocality lies in its out-of-focus light rejection capability. This aptitude is linked to the pinhole diameter with respect to the PSF size (the so-called $Airy\,Unit = \frac{1.22\lambda}{NA}$). Both background rejection value (using a Sandison *et al.* approach[33]) and lateral resolution have been computed, varying the pinhole size. The values have been normalized by regular-RCM values (with pinhole size set to 1 Airy Unit). Gold beads (60 nm diameter) in immersion oil were imaged (see Methods), the light back-scattered by the coverslip is acting as a source of background light. Opening the pinhole in the imaging path leads to a resolution increase until it reaches a maximum plateau at 2x improvement (Figure 3h). The background rejection, which directly affects the signal-to-noise ratio of the microscope, decreases concerning the pinhole diameter. We found an optimum at a pinhole diameter of 3 Airy Units: a 2x resolution gain and a background rejection preserved at 30% as compared to regular RCM is achieved (Figure 3h).

Metrological sample imaging

We have demonstrated that the resolution of RCM can be doubled in the lateral directions and enhanced by a factor of 1.5 in the axial direction without compromising the background rejection. To validate the measurements performed on punctual objects, 3D biological samples with metrological properties have been imaged. Fossil diatom shells immersed in water (see Methods) have been considered as biological resolution targets since they have quasi-fractal structures[34]. Figure 4a and Movies 1 and 2 (see *Extended data*)[35] show a 3D reconstruction of a diatom shell with both regular-RCM and the rescanned-RCM. The regular and rescanned-RCM images present notable differences: this is due to the overall 3D frequency support, which hugely differs between both modalities. The gain of resolution with rescanned-RCM is visible when using the rescanned-RCM (see in particular the insets in Figure 4a) and it shows how the small details of shells can be extracted and reconstructed in 3D. The gain in 3D (i.e. ($x \rightarrow\times 2$, $y \rightarrow\times 2$, $z \rightarrow\times 1.5$) is consistent with the own measured on beads.

We have studied the effect of at-depth imaging regarding the lateral resolution improvement of rescanned-RCM versus regular-RCM. Diatom shells in agarose gel (see Methods) were imaged up to the maximum depth authorized by our microscope objective (100 µm, Figure 4b and 4c). By computing the 2D MTF at different depths starting from the coverslip (z_0=0) up to 100 µm, we demonstrate that the resolution

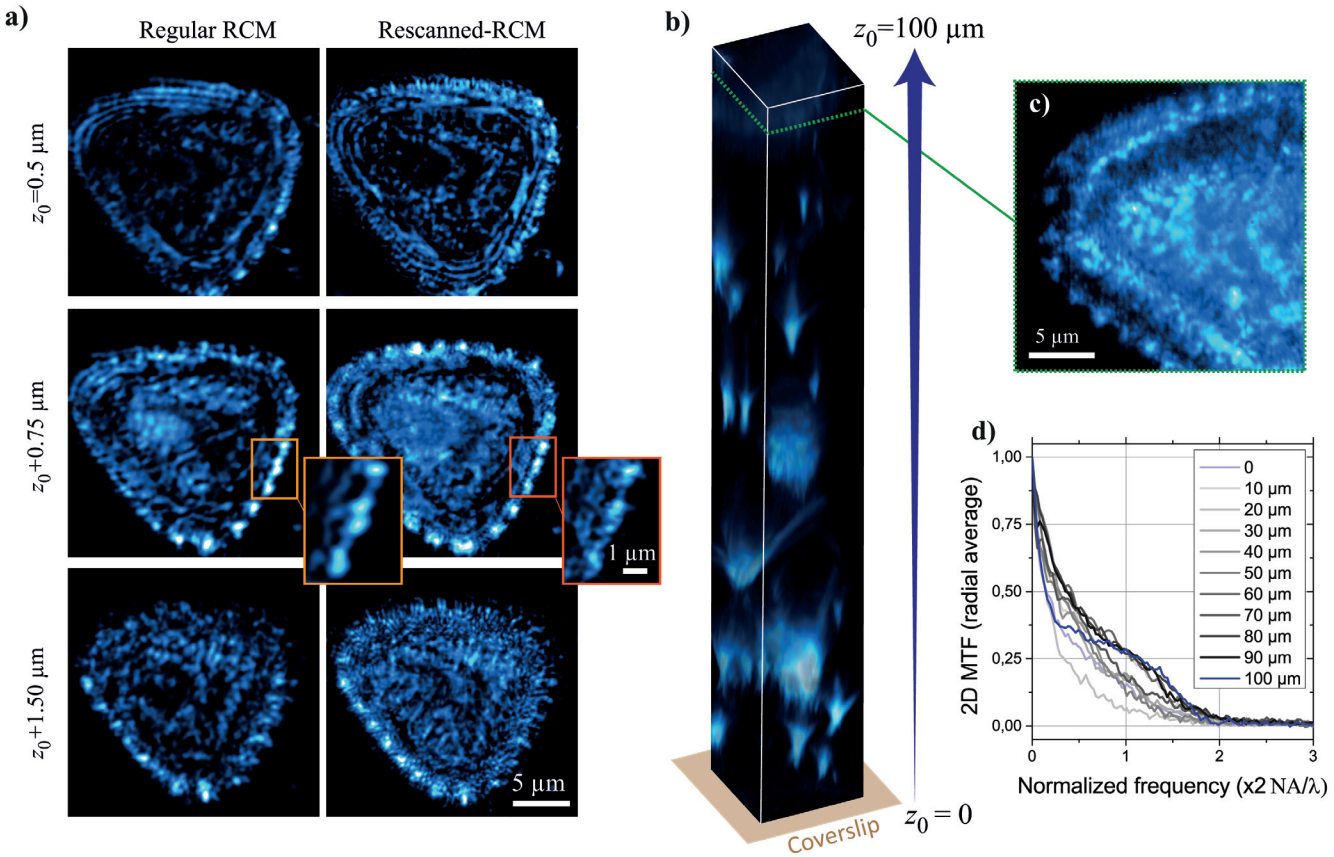


Figure 4. Diatom shells imaging in different planes. (**a**) From z_0 = 0.5µm (up) to z_0 + 1.5µm (down) imaged with regular reflectance confocal microscopy (RCM) (left) and rescanned RCM (right). (**b**) z-stack volume imaging of a diatom gel from z_0 = 0µm (coverslip) to z_0 = 100µm. (**c**) 2D image form ***(b)*** at plane z_0 = 95µm. (**d**) 2D MTFs measured on ***(b)*** at different depth from 0 to 100µm in the sample. See Videos 1 and 2 for complete 3D visualization of the diatom shell ***(a)***.

doubling granted by rescanned-RCM is preserved at depth in scattering samples (Figure 4d).

Rescanned-RCM can also be applied in nano-material sciences to perform metrological characterization of label-free samples with unprecedented optical resolution (imaging of 90 nm diameter silver nanowires without using non-linear properties, see *Extended data: Annex 2*)[31].

Live sample imaging

Finally, we applied rescanned-RCM to image living cells. Label-free adherent cells (mouse embryonic fibroblasts, MEF) have been observed and results are presented in Figure 5. For fast 2D imaging (temporal resolution of 1.25 Hz), we focus on a thin part of the cell, a lamellipodium at 1 µm above the coverslip and image over time. The laser power has been reduced to limit phototoxicity (laser power of 30 µW, average power over the field-of-view of 100 W/cm^2). Such temporal resolution with limited phototoxicity allows us to discriminate and follow organelles in their native environment during minutes (no visible effect of the laser on the sample after five minutes of constant imaging).

On a large field of view (Figure 5a and 5b) the lamellipodium and membrane edges are visible in label-free, both in RCM and quantitative phase imaging[36]. Some slowly varying fringes

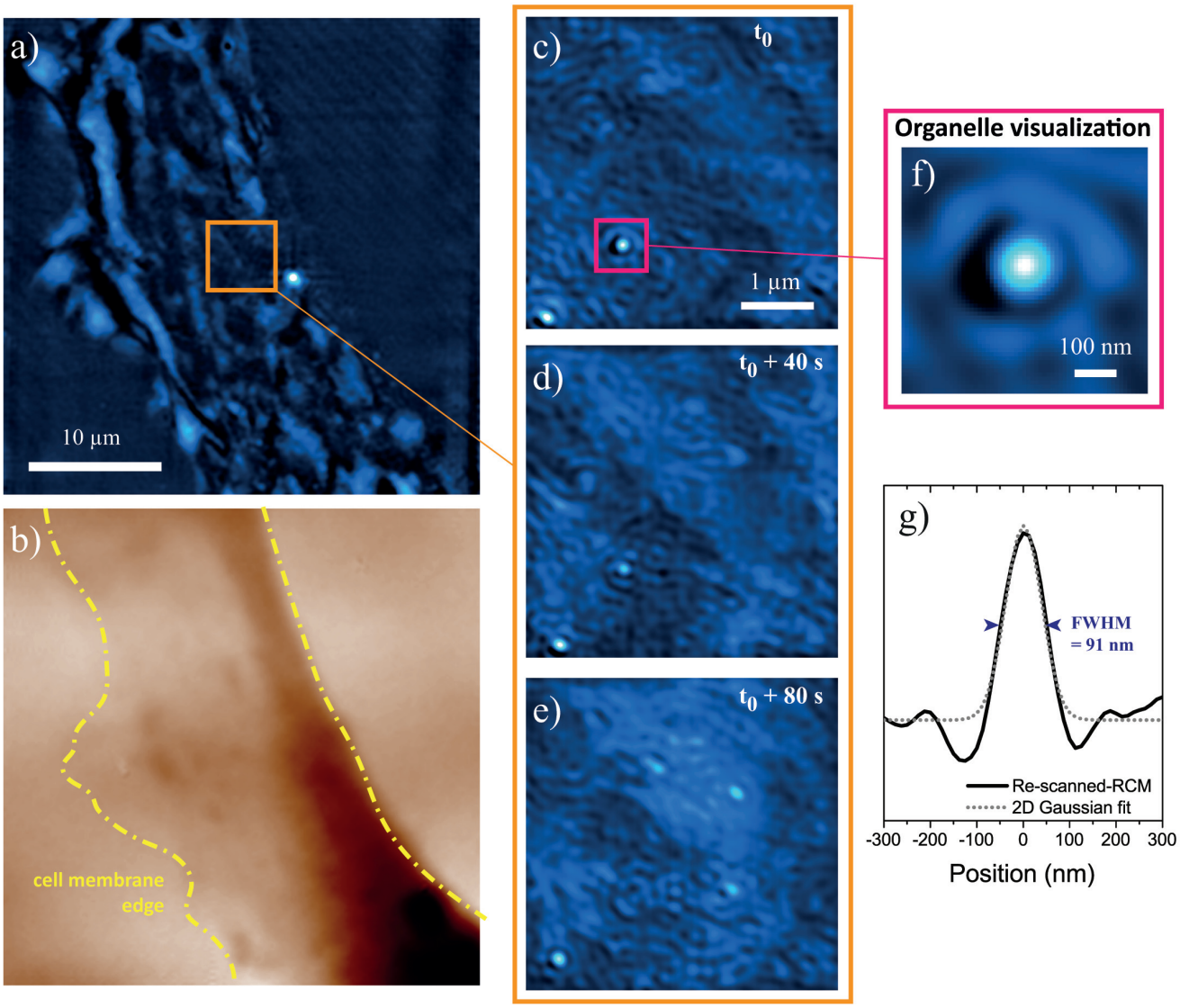


Figure 5. (**a**) Mouse embryonic fibroblast cell lamellipodium imaged with re-scanned reflectance confocal microscopy (RCM). (**b**) Same as ***(a)*** observed with quantitative phase imaging (the darker the look-up table, the longer the optical path). (**c**–**e**) Zoom on ***(a)*** at three different time points separated by 40s. (**f**) Zoom on a sub-structure of ***(b)***. (**g**) Line-out and 2D Gaussian fit of ***(f)***. All images are 2D-deconvolved. See Video 3 for the dynamic sample imaging.

are also visible in the images and are formed by the interferences between the light back-scattered from the top and the basal membrane on this thin part of the cell. Our improved resolution is key to detect and follow organelles with a very large signal-to-noise ratio (>100, Figure 5c–e). The diameter of the detected object has been measured down to approximately 90 nm (Figure 5f and 5g) and the non-spherical shape of some organelles such as mitochondria can be studied and followed in very crowded environments without using labeling and with limited phototoxicity.

Discussion

We demonstrated that CM can reach an unprecedented 3D resolution by combining photon reassignment (originally developed for fluorescence-based CM) with label-free reflectance imaging. 3D super-resolution imaging can be achieved with an efficient background rejection and at depth imaging capabilities. Our optical photon-reassignment setup unlocks optical resolution of $(r_x, r_y, r_z)_{@\lambda=445nm}$=(86,86,248) nm^3 without requiring any computation. This is a doubled lateral resolution and x1.5 resolution improvement in the axial direction regarding regular RCM. Currently, the only far-field optical techniques that surpass our resolution are based on fluorescence photophysics (including depletion and saturation) but at the price of high-phototoxicity or sparse-labeling (single fluorescent particle imaging). Long duration live sample imaging without constraint on the sample and/or medium can only be achieved with structured illumination microscopy[37] and are limited to > 100 nm resolution. Our resolution improvement beyond the 100 nm resolution barrier and without requiring neither fluorescence nor computation is a new step in biological sample imaging. It unlocks studies in very crowded environments such as a living cell and at any depth within the sample, where state-of-the-art label-free techniques such as interferometric scattering (iSCAT) microscopy[38] reach their limits. Our approach can be applied to any samples and we have demonstrated its efficiency not only on biological samples (diatom shells and living cells) but also on nanomaterial characterization without requiring near-field or electron microscopy.

Using on-the-shelves only components, we were able to form 5 × 5 µm^2 super-resolved images at 1.25 Hz. It has been demonstrated that the scanning speed of optical-reassignment-based methods can be tremendously increased using custom optical elements and a more challenging alignment[26,30,39,40]. Our rescanned-RCM is a cost-effective solution for any optical microscope, the overall price of this add-on being less than 4k€ (including the laser, scanners, and camera; see Methods). This study paves the way to fully-optical fast investigations of the intimate 3D nano-structure of any samples with limited invasiveness.

Methods

Experimental setup

A 445 nm laser diode (500mW, Newgazer Technology, Shenzhen, China) is spatially filtered through a 50 µm diameter P_1 pinhole (see Figure 6) and collimated onto a 2D scanning galvo-mirror system (GVS002, Thorlabs, USA) placed in a plane conjugated with the pupil of the microscope. The apochromatic microscope objective (UPLSAPO60XS2, 60x NA=1.3, silicon immersion, Olympus, Japan) focuses the light on the sample with a diffraction-limited *d*-size as shown in Figure 1. The back-scattered light is recollected by the system using the same optical path as illumination. After passing through a 50/50 non-polarized beamsplitter, the back-scattered light is filtered through a second pinhole P_2. The size of this pinhole is directly linked to the performance of the system as discussed in results and Figure 3. In our setup, we have used a 200 µm pinhole for optimal resolution and background rejection. The light passing through the pinhole is collimated and sent on a second 2D galvo-mirror system (GVS002, Thorlabs, USA) conjugated with the pupil plane. This re-scanning mirror system redirects the collected photon onto a low-cost CMOS camera (DCC1545M, Thorlabs, USA) with a scanning amplitude of twice the amplitude of the first scanning mirror system. To compare the performance of the reflectance photon reassignment scheme and an optimal regular RCM we used an arm (Figure 6) constituted of a 90/10 non-polarized beamsplitter, a pinhole P_3=50µm and a mono-detector (amplified photodiode). To perform z-scans, the microscope objective is moved by minimum increments of Δz = 50 nm (MFC-2000 Z-Axis Drive and Controller, ASI, USA). An ADC/DAC acquisition card (NIUSB-653, National Instrument USA) is used to tune the angular position of the 2D galvo mirrors (angular resolution of 15 µrad) and to digitize the amplified photodiode voltage.

Acquisition software and image processing

A homemade software written in Labview (NI LabView 2013, National Instrument USA) has been developed to drive the setup. The images don't require any further treatment to achieve super-resolution. However, for quantitative measurements, a deconvolution algorithm was applied to the raw images. The deconvolution algorithm is based on a classic Wiener filter applied considering experimental PSF and implemented in Labview. As an alternative, it is possible to use the DeconvolutionLab2 open-source software[41].

Sample preparation

Gold beads. For resolution measurements, 60 nm diameter beads were used acting as punctual sources (OD 1, stabilized suspension in citrate buffer, PubChem Substance ID 329765549, Sigma Aldrich). The beads were deposited by spin-coating on a plasma cleaned cover-slip. Objective immersion oil (Nikon) was added to quasi-match the refractive index of the coverslip and minimize its back-scattered light. For studies of agglomerated but resolved objects (Figure 2b–f), 100 nm diameter gold nanobeads were used (OD 1, stabilized suspension in citrate buffer, PubChem Substance ID 329766387, Sigma Aldrich). A droplet of this solution was dried on a coverslip at room temperature for five hours to create bead clusters. As for 60 nm beads, the dried sample was immersed in immersion oil to minimize the back-scattered light from the coverslip.

Diatom shells. The diatom sample was prepared from diatomaceous earth (*La Droguerie Ecologique* - SDEB ECODIS, France) -a siliceous sedimentary rock composed of fossilized

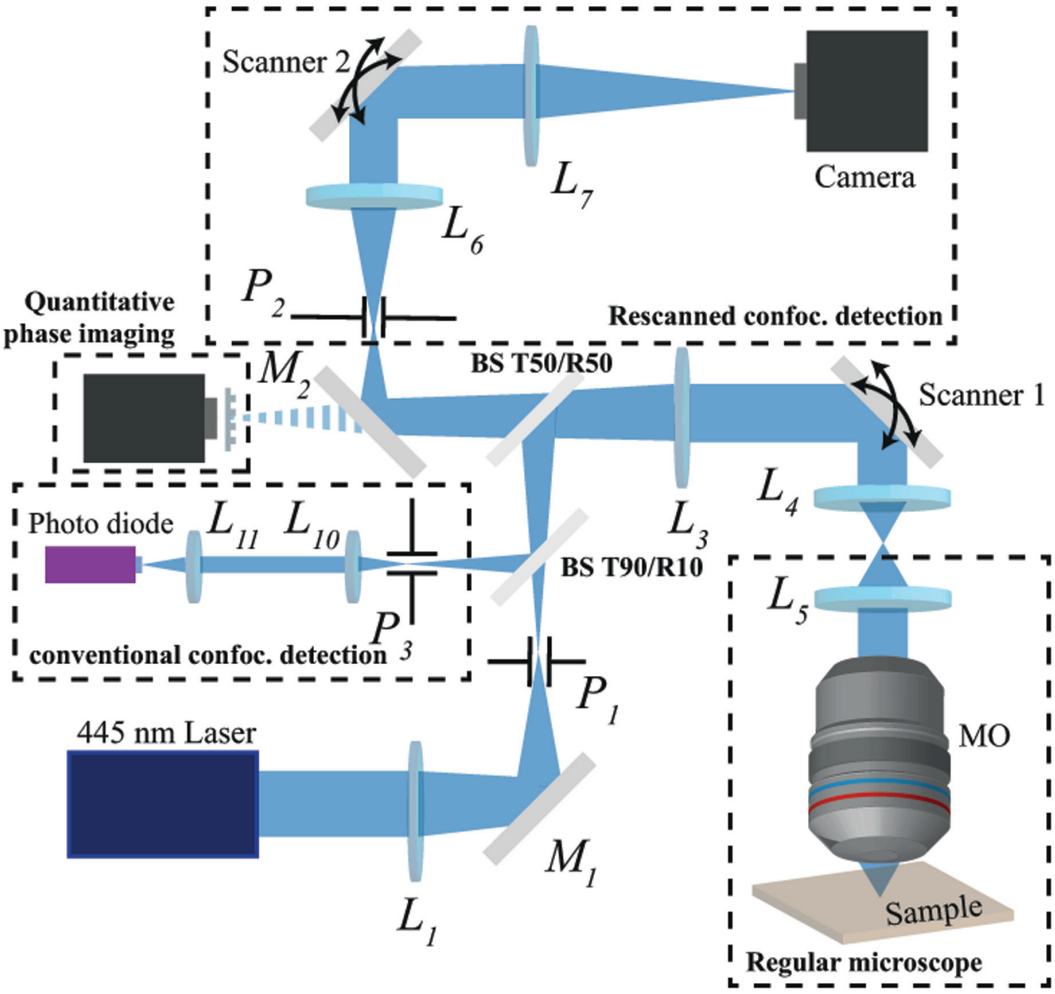


Figure 6. Complete optical setup overview. L_n are doublet lenses, M_n mirrors, P_n pinholes, *TX/RY X%/Y%* transmission/reflection beamsplitters, and scanners are 2D galvanometer scanners.

diatom shells- suspended in distilled water. The solution was spin-coated on a coverslip to have a uniform distribution of the diatom shells. The coverslips were immersed in distilled water to mimic biological sample imaging conditions. For thick 3D biological phantom composed of diatom shells, a 1% fossil diatom shell, 1% agarose (Low melting, Roche) solution in distilled water was prepared. The solution was then heated in the microwave 90 seconds so the agar could dissolve within the mixture. The gel was hot-deposited on a microscope coverslip. After cooling, imaging was performed.

Live cells. Wild type mouse embryonic fibroblasts (MEFs, source: courtesy of Arnaud Mourier lab). MEFs were isolated from mouse embryos of a pathogen-free C57Bl6/N mouse (Day 13.5) using classical trypsin procedure. Briefly, embryos were mechanically dispersed in presence of trypsin by repeated passage through a 1000 pipette tip and plated with MEF media (DMEM, 10% FCS, 1% nonessential amino acids, 1 mM L-glutamine, penicillin/streptomycin). Immortalization of primary MEFs was then achieved using classical viral transduction of pMX-LargeTcDNA expressing SV40 large T antigen. Cells were grown in DMEM high glucose (4.5 g/l) with L-glutamine and sodium pyruvate supplemented with 10% FBS, and 1% penicillin-streptomycin (Corning, USA) in a humidified cell culture incubator (37°C and 5% CO2). After several days, cells were plated at low confluency on coverslips. The rescanned-RCM imaging was performed at room temperature (24°C) in less than 30 minutes after taking the sample out of the incubator.

Data availability

Underlying data

Zenodo: Super-resolved Reflectance Confocal Microscopy data on Diatom shells and live MEF cells. https://doi.org/10.5281/zenodo.4421019[42].

This project contains the following underlying data:

- Diatom images in TIF format
- Live MEF cell images in TIF format

Extended data

Zenodo: Super-resolved Reflectance Confocal Microscopy 3D reconstruction of a Diatom shell. https://doi.org/10.5281/zenodo.4421193[35].

This project contains the following extended data: back-to-back the performance in term of resolution of both standard reflectance confocal microscopy and super-resolved rescanned reflectance confocal microscopy.

- FigureDiatom-Rotation.avi (Movie 1: 3D stack angular rotation)
- FigureDiatom-Zstack.avi (Movie 2: Zstack)

Zenodo: Super-resolved Reflectance Confocal Microscopy time-lapse imaging of a living MEF cell lamellipod. https://doi.org/10.5281/zenodo.4421233[43].

This project contains the following extended data:

- FigureLamellipod.avi (time-lapse of a label-free living Mouse embryonic fibroblast cell observed with super-resolved rescanned reflectance confocal microscopy)

Zenodo: Annex to the paper "Label-free super-resolution imaging below 90-nm using photon-reassignment". https://doi.org/10.5281/zenodo.4421353[31].

This project contains the following extended data:

- Annex 1: Theory of photon reassignment super-resolution
- Annex 2: Nanomaterial imaging

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

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The authors present an improvement of optical resolution in label-free reflectance confocal microscopy, by adapting a scheme similar to photon reassignment to label-free reflectance imaging, called rescanned-RCM. Interesting high resolution reflectance images are shown in model samples at large depths, with emphasis on the fact that this label-free modality allows long term imaging, even though quite high laser power is used.

The authors claim in the conclusion that the method “unlocks studies in very crowded environments such as a living cell and at any depth within the sample”, which is a bit oversold considering the specificities of the optical contrast measured. Indeed since the signal measured comes from reflectance from a structure, there are some stringent conditions on this structure to make it a valuable contrasted marker. A few aspects related to this are addressed below.

An important parameter limiting RCM imaging is first the scattering cross section of the imaged structure. This conditions the used laser power and affects the signal to background ratio. A rough calculation estimating how much scattering cross section is required from the structures imaged, in comparison to scattering from the rest of the sample, is necessary to evaluate the expected SBR and SNR ratios in typical biological samples.

Along the same lines, the assessment of the optical resolution is made on very bright scatterers within a homogeneous environment and it would be interesting to give a general idea of the degradation of this resolution when measured in biological conditions.

The metrological properties mentioned in the manuscript when it comes to imaging fossil diatom shells is not very clear: in what are the fractal properties of these shells helping to use these samples for metrological purposes, what is exactly the size of the smallest structure expected to be visible in reflectance?

Another important parameter possibly limiting RCM is the heterogeneity of the sample in depth. The large depth image shown in Figure 4b demonstrates a clear high resolution even at a 100µm

depth, however this is shown on a sample made of a sparse repartition of cells in a homogeneous agarose gel. The sample is claimed to be scattering, which is not quantified. Can the authors add a comment or a measurement on the performance of RCM in depth in a biological tissue? We expect a tissue to bring a higher level of heterogeneities and resolution loss at depth. An important application of RCM is for instance the visualization of collagen fibers in tissues, since they provide a high index mismatch contrast; would such approach work in such sample?

In figure 5 the fringes visible in the image are attributed to interferences between the basal and top parts of the cell membrane. It is actually not very clear what is the contribution of the back-reflected light by the sample surface in this image: since the basal membrane is very close to the coverslip, this reflected signal is also expected to play a role in the measured signal.

It is said that the resolution is key to detect organelles in the cell lamellipodium, however since these organelles appear as quite isolated objects, wouldn't they be visible as well in a standard RC image? The authors mention the possibility to observe non-spherical shapes of some organelles such as mitochondria, it is not very clear from the data shown.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Optical physics, microscopy.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 19 May 2021

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The authors describe an implementation of a rescan confocal microscope that performs all-optical pixel reassignment. The setup is nearly identical to that presented in Ref. 25. The difference here is that it is applied here to reflectance instead of fluorescence. In turn, reflectance rescan confocal microscopy has been previously described in Ref. 30, albeit using a more complicated setup. In other words, though there is little innovation in this paper, there is some value to using the simpler setup than in Ref. 30. The paper would benefit from more analysis. For example, it's interesting that the resolution improvement in Fig. 3h seems to be better than that for fluorescence imaging (e.g. see Sheppard *et al*. (2013)[ref-1]). Even the lateral resolution improvement is somewhat better than expected (sqrt(2)) . The authors should comment on this. Better yet, they should offer an explanation for this.

Minor comments:

1. The authors present 3D PSFs in Fig. 3. But the authors should bear in mind that reflectance confocal is not a linear imaging system. In other words, the imaging technically cannot be described by a PSF.

2. Is the kink in Fig. 4d something to be expected?

3. What is Fig. 5f supposed to represent? How do the authors know this is an organelle rather than a piece of dust.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microscopy.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 07 April 2021

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?

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The authors report about a label-free confocal reflection microscope which uses pixel reassignment (realized by re-scanning) for enhancing the lateral resolution by twofold and the axial resolution by 1.5 fold. Re-scanning microscopy is a special variant of Image Scanning Microscopy (ISM), which allows for doubling the lateral resolution in confocal microscopy, similar to what is done by structured illumination microscopy for wide-field microscopy. The core idea is to record the confocally detected signal not by a point detector, but by a wide-field imaging detector. Conventional ISM then applies dedicated algorithms to reconstruct form the totality of recorded images at each scan position a final enhanced-resolution image with doubled resolution. In re-scan microscopy, this algorithmic step is partially realized by rescanning collected emission onto a wide-field detector with appropriate scan rate. ISM and re-scan microscopy have been so fare mostly used for fluorescence microscopy, and lately for Raman microscopy, and the authors now extend this to reflection confocal microscopy (RCM). The advantage of RCM is that it does not require any labeling or specific Raman signals, but basically works with any sample that generates sufficient signal and contrast in reflection. The authors demonstrate that their instrument works close to the theoretical limit and indeed provides doubled lateral resolution and additional axially resolution enhancement by a factor of 1.5. This is certainly a very noteworthy achievement which deserves publication. However, I have a few comments and questions that should be answered prior to indexing:

1. The authors compare their method against different published realizations of ISM/re-scan microscopy and claim that their method yields "... instant super-resolution imaging even at depth (> 100 μm) without any computation ...." and they reclaim it again in the discussion by writing "Our optical photon-reassignment setup unlocks optical resolution of (rx, ry, rz)@ λ=445nm=(86,86,248) nm^3 without requiring any computation." But then they explain how they determine the resolution: "To quantitatively determine the actual resolution of the microscope and its imaging performance, we have computed the 2D MTFs from both re-scanned and regular RCM frequency supports (Figure 3g)." It seems that there is here a

fundamental misconception about how to estimate resolution enhancement, which leads to an unfair and unfavorable comparison between previously published work and the work presented here. The resolution enhancement is typically measured by directly measuring the point spread function (PSF) of a sufficiently small point-like object (usually small fluorescent beads, so-called PSF beads, in fluorescence microscopy), and then measuring the width of the PSF as a proxy for the achieved resolution. Moreover, as explained in detail in the original publications by Sheppard and by Müller & Enderlein, ISM (or re-scan microscopy) leads to a suboptimal PSF which can be, however, corrected by a re-weighting of the Fourier components of the ISM image in Fourier space. This explains why raw ISM images always show a lateral resolution enhancement of only ~sqrt(2), and only after appropriate Fourier reweighting they can come close to the theoretical limit of 2. The same should be also true for the re-scan RCM presented here. By only looking at the frequency support of the optical transfer function (OTF = Fourier transform of PSF), this rescan-induced PSF deformation is hidden, and of course, the OTF indeed shows the presence of Fourier components up to frequencies double as large as those of a pure RCM. But the latter is also true for all cited fluorescence and Raman ISM methods: The frequency support in all these methods is also doubled, however when measuring the width of the PSF, it only gives a ~1.4 fold enhancement (without Fourier re-weighting). Thus the current authors should compare width of PSFs, not simply measure frequency support, and moreover, a Fourier re-weighting (or more sophisticated Wiener filtering) should also lead to an improved image quality for their re-scan RCM, as it does for fluorescence or Raman ISM.

2. In the theoretical model presented in “Annex 1: Theory of photon reassignment super-resolution,” why are the integrations over x_0 in eq. (1) and x in eqs.(2) and (3) not extended over the whole three-dimensional space, why only across a two-dimensional plane? The sample and illumination are 3D, so light is generated everywhere and will contribute to the final image. What is the connection between the OTF of eq.(4) and the image function of eq.(3)? Where is the confocal pinhole taken into account? The OTFs of eq.(5) look like the OTFs for focused excitation alias wide-field detection with no pinhole. I strongly recommend that the authors consult the appendix of O. Schulz *et al*. [1] where the full theory of confocal ISM is presented (which will be fully applicable to re-scan RCM).

3. On page 6, the authors write “We experimentally determined the axial resolution of the re-scanned RCM to be r_z{rsRCM} = 248 ± 8 nm as compared to the regular RCM theoretical axial resolution of r_z{rsRCM} … = 373 nm. This gain of 1.5 ± 0.05 is consistent with the experimental gain measured at 1.4 ± 0.1.” But the reported values are experimental values? How can they be consistent with the experimental gain? The experimental values are consistent with the experimental values?

4. Can the authors explain what the theoretical foundation of the observed resolution enhancement along the optical axis is? As explained and numerically shown in the above mentioned paper by Schulz *et al*., one would not expect an axial resolution enhancement form ISM/re-scan for a well-adjusted confocal microscope.

5. In Figure 4g, what do negative values of the 2D MTF mean?

6. In Figure 4h, how is resolution determined? Again via frequency support of the OTF/MTF?

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PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Super-resolution microscopy, single-molecule spectroscopy, nano-optics, plasmonics.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.