Optical Imaging Innovations for Atherosclerosis Research
Multiphoton Microscopy and Optical Nanoscopy

Remco T.A. Megens, Mariaelvy Bianchini, Martin M.N. Schmitt, Christian Weber

Abstract—Cardiovascular disease is the leading cause of death and morbidity worldwide. Improving vascular prevention and therapy based on a refined mechanistic pervasion of atherosclerosis as the underlying pathology could limit the effect of vascular disease in aging societies. During the past decades, microscopy has contributed greatly to a better understanding of vascular physiology and pathology by allowing imaging of living specimen with subcellular resolution and high specificity. An important advance has been accomplished through the application of multiphoton microscopy in the vascular domain, a technological development that enabled multidimensional and dynamic imaging deep into the cellular architecture of intact tissue under physiological conditions. To identify and validate new targets for treating atherosclerosis, novel imaging strategies with nanoscale resolution will be essential to visualize molecular processes in intracellular and extracellular compartments. This review will discuss the current use of 2-photon microscopy and will provide an overview and outlook on options for introducing nanoscopic optical imaging modalities in atherosclerosis research.

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Key Words: atherosclerosis ■ microscopy

Atherosclerosis is a chronic inflammatory disease of medium and large arteries characterized by the intimal thickening, fibroproliferative progression, and development of unstable plaques, which can lead to relevant clinical events, such as myocardial infarction and stroke. Therefore, a detailed understanding of the underlying structural, cellular, and molecular alterations is of primary importance and studies aimed at direct visualization of these processes may contribute to the development of novel preventive, diagnostic, and therapeutic approaches. Hence, imaging technologies are required that allow for morphological and functional investigation at high spatial and temporal resolution deep in living tissues.

Two-photon laser scanning microscopy (TPLSM) or multiphoton microscopy was introduced in the beginning of the 90s as a new optical fluorescence technology. It exploits powerful femtosecond-pulsed lasers for the nonlinear excitation of fluorophores (either exogenous fluorescent dyes or endogenous autofluorescent tissue components) by 2 almost simultaneous (10^{-15}) s photons with half the energy (double the wavelength) of those used for single-photon excitation in conventional confocal microscopy systems. Because the longer wavelength light used for 2-photon excitation (typically 800–1000 nm) suffers less from scattering and absorption features, the latter facilitates imaging of whole-mount tissues, such as intact viable arteries. In contrast, well-tuned confocal microscopes offer an acceptable signal-to-noise ratio and thus good quality imaging only within the first 50 μm of depth in large arteries. Furthermore, appropriate use of low-energy light also accounts for reduced photodamage, which is beneficial for in vivo experiments. Finally, because 2-photon excitation is a low probabilistic event, only in-focus illuminated regions of the specimen achieve the high density of photons required for excitation to occur. As a result, excitation and fluorescence emission are confined to a small defined volume within the focal plane, resulting in optical sectioning, z-stack collection, and 3-dimensional (3D) reconstruction of the sample without the need for a pinhole to exclude the contribution of out-of-focus light to image formation as in confocal laser scanning microscopy (Figure 1A). The absence of a pinhole blocking part of the emitted fluorescence accounts for greater sensitivity and improved signal-to-noise ratio in TPLSM, especially for highly scattering tissues and organs.

TPLSM in Atherosclerotic Research

As atherogenesis primary involves medium and large arteries, high-resolution optical imaging of the arterial wall structure in healthy and diseased vessels can provide unique information when compared with other investigation methods, such as...
as histology. A method that enables TPLSM in ex vivo intact murine carotid arteries mounted and pressurized in perfusion chambers and where both intraluminal and extraluminal physiological conditions are maintained for prolonged time has been used for studying of structural components of the intima, such as endothelial cell orientation and activation, and endothelial junction distribution, and glycocalyx thickness. TPLSM can also be used for studying the extracellular matrix proteins collagen and elastin, 2 key mechanical components present in these layers. Collagen can be visualized by second harmonic generation, a nonlinear energy-conservative process based on the recombination of 2 near-infrared scattered photons into one with exactly half the wavelength. This phenomenon only occurs in dense noncentrosymmetric samples, such as fibered collagens and striated muscle myosin, and produces mainly forward scattered light in ≤200-μm thick specimens, whereas backward scattered photons are the major signal components at deeper locations in tissues. This strategy has been used to characterize the collagen fiber network in the tunica adventitia and intima of mounted healthy and diseased arteries and to evaluate plaque burden and the thickness of the fibrous cap of atherosclerotic stable and unstable plaques in the human aorta. Elastin is the most abundant extracellular matrix protein in the tunica media where it forms elastic laminae interspersed among smooth muscle cells. When illuminated with near-infrared light, it emits autofluorescent signals in a broad range of the visible spectrum, providing further useful insights into arterial structure and function.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>3D</th>
<th>3-dimensional</th>
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<tr>
<td>STED</td>
<td>stimulated emission depletion</td>
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<td>TPLSM</td>
<td>2-photon laser scanning microscopy</td>
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Figure 1. A, Single photon vs. 2-photon excitation, with single-photon excitation fluorophores excited throughout the complete beam path, resulting in emission above and below the focal spot. With 2-photon excitation, fluorescence emission is restricted to the focal spot thereby achieving depth discrimination in the absence of pinholes (as in confocal microscopy). B, Extended depth of field projection demonstrating myeloid cells (green) crawling in close contact with the carotid artery wall in vivo (scale bar, 25 μm). Tetra-methylrhodamine-dextran (orange) was used to label the blood stream. Total z-depth of the extended depth of field projection is ≈18 μm (step size, 2 μm). C, Tracking of myeloid cells (depicted in the red box) over time using 3-dimensional (3D) isosurface rendering derived from 3D 2-photon laser scanning microscopy (TPLSM) stacks over time. Grey arrow (B) indicates the direction of blood flow. D, 3D reconstruction of a TPLSM stack of the same data set, where the viewing angle was altered to mimic a transversal view on the vessel wall and adherent cells (green), collagen in the tunica adventitia (blue), and the lumen (red). Adapted from Chêvre et al with permission of the publisher. Copyright ©2014, Wolters Kluwer Health. Authorization for this adaptation has been obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.
Atherosclerosis features complex interactions between dysfunctional endothelium and intraluminally recruited leukocytes. To fully understand the dynamics of these events, in vivo imaging of the large arteries and the heart in preclinical models is essential. Yet, this approach is not only limited by the necessity to penetrate into thick scattering tissues but also by the cyclic movements of the specimen because of both heart and respiration rates (in mice 5–6 Hz and 2–4 Hz, respectively). TPLSM is the optical microscopy technique of choice for deep high-resolution imaging, especially when implementing the system with an optical parametric oscillator, thus dramatically increasing the signal/noise ratio. However, when it comes to moving samples, the necessity of point scanning causes major problems, namely in-frame and between-frame motion artifacts. The former are image distortions because of the movements occurring in the time window between the first and the last scanned point of the same picture, whereas the latter is the cause of the acquisition of different focal planes in subsequent images. As a result, it is difficult to track cells and perform functional studies under these conditions. Video-rate acquisition (>30 Hz) can be achieved with resonant or parallelized scanners, with little consequences on the overall image quality and resolution. Moreover, strategies to mechanically stabilize moving samples after surgical exposure and timed image acquisition have been used. Image acquisition triggered on respiratory/heart cycles, mechanical stabilization, and strong fluorescent markers enabled recording of good quality time-lapse movies of the arterial wall and leukocyte interactions with the vessel wall, even in 3D (Figure 1B). Using a comparable layout, Li et al managed to track single leukocytes recruited to damaged cardiac tissue in living mice after coronary ischemia reperfusion, both in transplanted and in native hearts, ≤120-μm depth. More recently, an intravital live cell–triggered imaging system has been developed by Ley et al to reveal monocyte patrolling and macrophage migration in atherosclerotic arteries, implementing cardiac triggered acquisition, as well as frame selection and image registration algorithms, to produce stable movies of myeloid cell movement in atherosclerotic arteries in live mice. TPLSM contributed to cardiovascular research by gaining new insights into the early stages of atherogenesis, plaque progression, and thrombus formation. Importantly, new information has been derived about leukocyte recruitment and function in atherosclerotic lesions. For example, plaque vasa vasorum permeability to adhering and transmigrating leukocytes has been investigated, and the role of endothelial junction adhesion molecule-1 in promoting monocyte influx into the arterial wall has been elucidated. Although monocytes and macrophages are thought to be the main leukocytes involved in atheroprecession, recent data indicate that neutrophils may play a major role at early time points. In monocyte-depleted Lysmyg−/− mice, it was possible to visualize neutrophils adhering at the carotid bifurcation and to track their transmigration from the lumen into the subendothelial space. This implies a transluminal route for the access of these cells to early sites of atherosclerosis where they promote monocyte recruitment and lesion development. At a similar stage of disease, neutrophil extracellular traps were found at the bifurcation sites of carotid arteries in vivo but not in healthy mice. The potential contribution of dendritic cells has been established in CC chemokine ligand 17 (CCL17)y/w Apoe−/− mice where CCL17-expressing dendritic cells were shown to accumulate in the lesion and to interact with T-cells, thereby promoting atherogenesis. Finally, TPLSM has proved to be a useful tool for investigation in different models of arterial thrombogenesis, either after chemical and mechanical or laser-induced arterial damage. Real-time platelet adhesion on exposed collagen, fibrin/fibrinogen deposition, and effects of antithrombotic drugs have been successfully monitored. The unique characteristics of TPLSM together with the ongoing development of the optical equipment and the preparation methods allowing physiological imaging have so far unveiled multiple cellular and molecular mechanisms of atherosclerosis and will certainly continue to evolve in the years to come. During the past decade, light sheet microscopy, in particular selective plane illumination microscopy, has been developed as an important tool for in vivo imaging in zebrafish or fruit flies, offering optical sectioning, fast image acquisition, and low toxicity. With appropriate sample preparation, selective plane illumination microscopy may evolve as an alternative for high-resolution imaging of ex vivo whole-mount (atherosclerotic) arteries.

Atherosclerosis Research: Toward Optical Nanoscopy

Despite recent progress, current optical techniques, including TPLSM and confocal microscopy, and other imaging modalities do not offer the resolution to gain access to the intracellular environment (<0.25 μm) in live tissues or are not applicable to physiologically relevant models of atherosclerosis. During the past years, ground-breaking optical super-resolution microscopy systems (also known as optical nanoscopy or diffraction-unlimited microscopy) have been developed enabling optical imaging with much higher resolving power than ever expected. In fact, beyond multiple publications in high-impact journals in the past decade, nanoscopic modalities have been considered so revolutionary that their creators have recently been awarded the Nobel Prize in Chemistry 2014.

Optical nanoscopy overcomes the diffraction limit of conventional optical microscopy as described in 1873 by Abbe. Abbe showed that by using lenses and imaging distances much greater than the wavelength of light, one can distinguish lateral distances ds=0.61λ/λsinθ, where λ is the wavelength of the light used and θ equals the numeric aperture of the lens. This also accounts for point scanning microscopy using laser excitation sources, such as confocal microscopy or TPLSM, where, because of the diffraction limit, it is impossible to focus a laser beam to a smaller spot than λ/2. As a consequence, objects closer than ~200 nm in lateral and ~450 nm in axial direction cannot be separated anymore with diffraction-limited optical microscopy. Thus, although modalities, such as confocal and TPLSM, are challenging the diffraction barrier by optimizing optical pathways and excitation principles, they do not break Abbe’s criterion.

Optical nanoscopy (diffraction-unlimited microscopy) advances the resolving power into the nanometer range (5–100 nm), a range that until recently was only accessible with electron microscopy or scanning probe microscopy. All nanoscopic modalities make use of the principle of...
fluorescence microscopy and the control of temporal on/off switching of fluorescence emission. This principle is applied in an organized matter by making use of (parallelized) point scanning microscopy (ensemble nanoscopy; Figure 2A) or randomly (stochastic nanoscopy; Figure 2B). Yet, although the importance of optical nanoscopy for biology has already been demonstrated, only little is known about its applicability for imaging atherosclerosis. We will thus mainly discuss the basics of the 2 Nobel prize–awarded nanoscopic methods and elaborate on the potential of these nanoscopic modalities for cardiovascular disease.

**Stochastic or Localization Nanoscopy**

Betzig and Moerner have been awarded the Nobel Prize in Chemistry 2014 as the driving forces for the development of localization microscopy. Localization-based fluorescence nanoscopy requires photoactivatable, photoswitchable, or dark triplet state generation of fluorophores. Fluorophores are switched on (bright) and off (dark) stochastically between bright and dark emission states by exposure to light of a specific spectrum. At each time point, only few molecules are on and imaged. Repeating this sequentially results in a sequence of thousands of images, each of fluorophores at a different location, which are subsequently superimposed to generate a single nanoscopic image. In the initial development of localization nanoscopy, the spectral properties of fluorophores were only delivered by specialized dyes or certain (destructive) sample preparation techniques, but nowadays, also standard fluorophores at ambient temperature can be used. Many variations of localization techniques have been developed, for example, photoactivation localization microscopy (PALM), fluorescence PALM, ground-state depletion microscopy, spectral precision distance microscopy, stochastic optical reconstruction microscopy, or 4D direct stochastic optical reconstruction microscopy. Lateral (and axial) resolution is dramatically improved in these techniques and is basically determined by the accuracy of detecting the location of single, optically isolated molecules, which is achieved with wide-field optics and highly sensitive electron multiplying charge-coupled device cameras or complementary metal–oxide–semiconductor arrays.

**Figure 2.** Nanoscopic strategies for on and off switching of fluorophores in a sample. **A.** Ensemble nanoscopic stimulated emission depletion (STED) principle where an excitation (green) and depletion donut laser (red) are overlapping and scanned (black arrow) over the sample. Only fluorophores in the center of the excitation laser emit light (state A), whereas molecules located in the depletion ring are switched off (state B) by stimulated emission depletion. The more powerful the depletion laser, the smaller the center spot will become. **Bottom,** Extended depth of field projection of collagen type I fibers in a commercially available suspension of collagen isolated from equine tendon (Takeda, Austria) regularly used for platelet adhesion studies, labeled with anticollagen type I antibody conjugated with AlexaFlour594 (invitrogen) and visualized with confocal microscopy (left) or 3-dimensional STED nanoscopy (right) depletion at 660 nm. **B.** In the stochastic switching mode, individual fluorophores are switched on (to state A) randomly in space, whereas the surrounding molecules remain in the dark state B. The distance between the on molecules should be >\(\frac{\lambda}{2\sin(\alpha)}\) to recognize individual molecules. Their coordinates are determined by calculation of their centroids. **Bottom,** Comparison of a conventional wide-field image with a stochastic (ground-state depletion microscopy [GSDIM]) image of microtubules (green) and peroxisomes (red) in mammalian cells. In the stochastic variant GSDIM, the molecules are first switched off (B) and then pop up spontaneously in (A). Scale bar, 1 μm. Adapted from Hell and Fölling et al with permission of the publisher. Copyright ©2008 and 2008, Nature Publishing Group. Authorization for this adaptation has been obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.
A lateral resolution of 10 to 30 nm is typical, but as little as 1 nm has been achieved. Thus, localization microscopy is especially useful for determining the exact sites of intracellular molecules and their interaction in fixed single cells, but also to evaluate molecule dynamics in vivo. Using 2-color PALM, Hsu and Baumgart were able to quantify different stages of protein-protein interactions in T-cell signaling microcluster of fixed samples. They showed that a decreased interaction of the kinase Zap70 and the adaptor SLP-76 in the cell periphery was correlated with reduced microcluster phosphorylation levels (Figure 3). For in vivo imaging, however, the overall requirements are more demanding, particularly about acquisition time. Because only a small subset of fluorophores is switched on and imaged at any given time, the acquisition of the entire sample usually requires thousands of images. This parameter determines the total acquisition time per sample. As such, the stochastic methods in general are rather slow because many frames need to be acquired to reconstruct a single super-resolution image. Still, Hess et al have succeeded in detecting photoactivatable green fluorescent protein-tagged hemagglutinin on the membrane of fibroblasts, and living cells expressing photoactivatable mCherry have been visualized using 2-color PALM. A study performed by Jones et al provided 2- and 3-dimensional images of clathrin-coated pits and their cargo transferrin in live cells with a lateral resolution of 25 to 30 nm and an axial resolution of 50 nm at an intriguing image acquisition rate of 0.5 to 1 Hz for 3D datasets (Figure 4). To achieve such fast- and high-resolving imaging, fast-switching dyes were combined with a strong reduction of the number of independent snapshots.

Figure 3. Photoactivation localization microscopy (PALM) nanoscopy of Zap70-tdEos (red) and SLP-76-dronpa (green) microcluster. A, Comparison of subcellular 2-color PALM (left) and total internal reflection micrographs (right), indicating that PALM nanoscopy is able to reveal different spatial interactions of Zap70 and SLP76. Scale bar, 500 nm. B and D, Higher magnification of boxes in (A). Zap70/SLP76 interaction was quantified using Manders colocalization coefficient (MCC) M1 and M2, where 1 resembles total and 0 no overlap. B, Broad colocalization with high MCC values (M1=0.71 and M2=0.73). C, Medium range colocalization with medium MCC numbers (M1=0.6 and M2=0.26). D, Low colocalization with MCC values near 0 (M1=0.21 and M2=0.08). Scale bar, 250 nm. Adapted from Hsu et al.

Ensemble Nanoscopy

Ensemble nanoscopy is often based on point scanning systems, such as confocal microscopes, or parallelized scanning microscopy. The best known modalities of ensemble microscopy are stimulated emission depletion (STED), for which its inventor Hell has been awarded with the Nobel Prize for Chemistry 2014, the related reversible saturable optical fluorescence transitions and structured illumination microscopy. Like TPLSM, STED depletion makes use of nonlinear optical principle where fluorescence intensity in the donut area decreases with increasing depletion laser intensity. In STED, the emission signal is produced from a much smaller volume than in confocal microscopy. This is achieved by overlapping the laser scanning illumination and excitation pulse with another high-power, doughnut-shaped, and longer wavelength laser beam, also called depletion laser. The center of the doughnut is overlapping with the center of the excitation beam. In the filled part of the doughnut, the molecules are forced back to the ground state (emission depletion), so that effective fluorescence emission, produced by the illumination laser, is only existent in the empty part of the doughnut. The size of the empty part is dependent on the power of the depletion laser, and in principle, it can be made infinitely small, thereby strongly improving the lateral resolution. Recently, it has been shown that the axial resolution of STED can also be improved using the same principle. When compared with confocal microscopy, a >175-fold smaller excited volume can be achieved by combining the horizontal donut laser with a second vertical donut-shaped laser line derived from the same laser source by means of phase tuning, which limits the excited volume in the direction. Home-built STED microscopes have already achieved lateral resolutions of <10 nm. The current commercial STED systems offer lateral resolutions of ~20 to 40 nm and axial resolution of ~100 nm. STED enables video-rate acquisition speeds (using resonant scanning), and acquisition rates of >80 Hz have been achieved. Because STED enables nanoscopic resolution purely based on optics, no postprocessing of diffraction-limited data is required, as is the case with other nanoscopic modalities. However, with deconvolution algorithms, the image quality and overall resolution can be further enhanced. In cardiovascular research, STED has been used to study various properties of cardiomyocytes, the cell membrane, thrombocytes, glycolipid-enriched microdomains in neutrophils, and flow-dependent upregulation of vascular endothelial cadherin clustering in vascular endothelial cell junctions. However, STED has not yet been used to study inflammatory processes and the nanostructure in atherosclerosis. Especially, studying of intracellular signaling pathways and intracellular and extracellular colocalization properties will strongly benefit from the enhanced STED resolution and multichannel properties. An alternative ensemble microscopic modality is structured illumination microscopy although the diffraction barrier is actually not broken as with localization or STED microscopy and the gain in resolution is less great. With structured illumination microscopy, the fluorescent probes are illuminated with patterned excitation light of saturating intensity (Moiré fringe patterns). Combined with sophisticated mathematics, a resultant image can be reconstructed with a resolution improved by approximately a factor.
Structured illumination microscopy is a versatile technique that is compatible with most standard fluorophores resulting in a less restrictive sample preparation. Moreover, image acquisition can be relatively fast, which is important for visualization of dynamic processes.

**Outlook of Optical Nanoscopy for Atherosclerosis Research**

Optical nanoscopy is a quickly expanding field, with many modalities becoming commercially available. The higher costs of such systems are a prerequisite for implementation in a more biological environment because they offer turn-key functionality, thereby enabling a multiuser environment and studying of complex biological samples without having to continuously adapt the hardware. At present, most commercial techniques have an advanced, flexible layout and offer tailored solutions with multi-channel and multidimensional properties. Development of next generation detectors or cameras with improved sensitivity will further advance the application of optical nanoscopic modalities. This is especially true for studying fast dynamic events where fast acquisition speeds are a prerequisite. The latter is currently often limited by the little fluorescent signals that can be derived from the small excitation volumes or single-molecule imaging, and as a consequence, prolonged acquisition times are required to achieve an acceptable number of light photons for enabling proper image reconstruction. Furthermore, much of the future improvements in optical nanoscopy are also expected to come from the ongoing development of dedicated fluorophores for each modality because their properties are a main determinant for imaging quality. Moreover, development of fluorescent labels that can penetrate into living cells will accelerate the application of nanoscopy for live-cell imaging.

For nanoscopic imaging of functional processes in living cells, stochastic techniques are currently limited because of the intrinsically low acquisition rates. Hence, stochastic nanoscopy is better suited for studying nonmoving fixed cells or slow dynamic processes where its high-resolution levels can be fully deployed. Yet, the limitations of lower acquisition rates may be solved by the ongoing development of faster switching fluorophores, novel sample preparation procedures, and by reducing the number of required single images. Ensemble-based STED nanoscopy facilitates video-rate image acquisition. Nonetheless, in practice, achievable acquisition rates merely depend on the number of detectable fluorescent photons within the acquisition window. On the other hand, the requirement of high-power depletion lasers for STED is a disadvantage for studying viable biological samples because of the risk of laser-induced side effects (photodamage or photoactivation). However, time-gating of the detected signals may help in reducing these risks.
STED signal has reduced the need for high laser power with preserved resolution. Combined with the availability of red-shifted (pulsed) depletion lasers and bright STED fluorophores, the potentially damaging effects of STED on the sample have been further limited. The continuing development of reversible saturable optical fluorescence transitions, which uses the STED principle, albeit at much lower laser powers by means of photo-switchable fluorescent proteins, will further reduce the probability of laser-induced damage and as such offers great potential for biological research. Finally, commercial STED systems are based on advanced confocal microscopes. Although STED is superior compared with confocal microscopy, it remains still a valuable modality for routine imaging experiments.

In conclusion, optical nanoscopy for atherosclerosis research offers the resolution to study the intracellular environment of living samples, a potential that will possibly generate an even bigger effect on atherosclerosis research than the introduction of TPLSM in the 90s. The power of optical nanoscopy has now become accessible for biological research because of flexible, stable hardware and suitable fluorophores. In the upcoming years, the strongly expanding field will further accelerate the usability of nanoscopy for atherosclerosis research.

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References
fluorophores. In the upcoming years, the strongly expanding field will further accelerate the usability of nanoscopy for atherosclerosis research. Breaking optical nanoscopy (super resolution) systems offer the resolution to study the intracellular environment of living samples, a capability methods allowing physiological imaging, multiphoton microscopy will continue to evolve atherosclerotic research in the years to come. Ground-breaking optical nanoscopy (super resolution) systems offer the resolution to study the intracellular environment of living samples, a capability applications: new insights into cellular processes on the nanoscale. Chemyphys. 2012;13:1986–2000. doi: 10.1002/cphc.201100986.


Significance

This review provides an overview of the basics and applicability of multiphoton microscopy and the Nobel-prize awarded optical nanoscopic modalities to further explore their potential for atherosclerosis research. The unique characteristics of multiphoton microscopy have already unveiled multiple cellular and molecular mechanisms of atherosclerosis. Together with the ongoing development of optical equipment and the preparation methods allowing physiological imaging, multiphoton microscopy will continue to evolve atherosclerotic research in the years to come. Ground-breaking optical nanoscopy (super resolution) systems offer the resolution to study the intracellular environment of living samples, a capability that will very possibly generate an even bigger effect on atherosclerosis research than the introduction of multiphoton microscopy. Moreover, the power of optical nanoscopy has recently become accessible for biological research because of flexible, stable hardware and a range of suitable fluorophores. In the upcoming years, the strongly expanding field will further accelerate the usability of nanoscopy for atherosclerosis research.
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