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Focal Molography – an optical method for label-free detection of biomolecular interactions

Christof Fattinger, Roche Innovation Center Basel, Andreas Frutiger, Inno Biotech AG & ETH Zürich

Abstract:

Focal molography is a new method for label-free molecular interaction analysis in crude samples. In contrast to refractometric optical sensors, focal molography is insensitive to nonspecific molecular interactions. This unique property is achieved with a special 2D nanopattern of molecular binding sites on a chip, termed mologram. A mologram is designed such that molecules bound to it diffract light constructively into a focal spot. The intensity of the focused light is measured to quantify the amount of bound molecules. In biological samples, highly abundant off-target molecules readily adsorb to the surface of the sensor. Yet, this process is completely random and the off-target molecules do not bind to the ordered binding sites of the mologram. Thus, their scattering is uniform in all spatial directions and therefore they hardly contribute to the measured light intensity in the narrow solid angle of the focal spot.

1. Introduction

Specific biomolecular interactions in crowded environments are central to life on the molecular scale. Label-free biomolecular interaction analysis is widely used for the study of biomolecular interactions and processes [1, 2]. The detected biomolecular interaction is directed by a molecular recognition [3, 4]. The recognition leads to specific binding of a distinct biomolecule to another distinct biomolecule [3, 4]. Ideally, we would like to observe the two molecules during complex formation. We can see such dynamic phenomena of large individual biomolecules in highly dilute solutions [5]. Yet, in crowded solutions this is impossible. The only way to see dynamic phenomena in real-life, crowded biological environments is to observe the interactions amongst two ensembles of biomolecules. To achieve this, we use the coherent detection of the refractive index changes that are induced by the spatial immobilization of biological matter through molecular interactions. This physical detection principle is termed “focal molography” (“molography” in short) [6, 7, 8]. In this article, we explain focal molography, its implications and some of its possible applications.

In short, molography is a new analytical method for the robust and sensitive label-free detection of biomolecular interactions without the use of additional fluorescent labels [6, 7, 8]. Its working principle is illustrated in Figure 1. A mologram is a coherent assembly of binding sites on a chip that form the blueprint of a diffractive lens. Biomolecules that bind to the mologram diffract laser light into a diffraction-limited focal spot, the focus of the mologram. The diffracted intensity in the focal spot correlates quadratically with the adsorbed mass and hence with the number of biomolecular interactions on the mologram. The focal spot monitors the collective binding activity on the entire coherent assembly. Molography is therefore a technique to directly “see” an ensemble of molecules in action.

Being able to see biomolecular processes unfold in real time allows us to understand the mechanisms of life as well as disease. As an ensemble, we envision a very small amount of one type of a biological molecule – the specimen – typically a few pg or less. The specimen shall be detected by a specific biomolecular interaction. This is achieved by the binding of the molecules in the specimen to predefined binding sites through a specific biomolecular interaction. The formation of molecular recognition complexes can be detected optically because biological matter has a significantly higher refractive index than the surrounding aqueous solution, which is mainly water [9]. A biomolecule in water is therefore a pure phase object. Frits Zernike already realized that it is the phase retardation of light induced by the specimen – not the absorption of light [10] – that is the relevant physical quantity for observation of a small biological specimen under the microscope. Based on this insight, Zernike introduced phase contrast microscopy in 1934 [10, 11]. Upon complex formation, the local refractive index increase induced by the binding of molecules leads to an increase in the scattering strength of the individual molecular recognition complexes. However, as mentioned above the signal of an individual

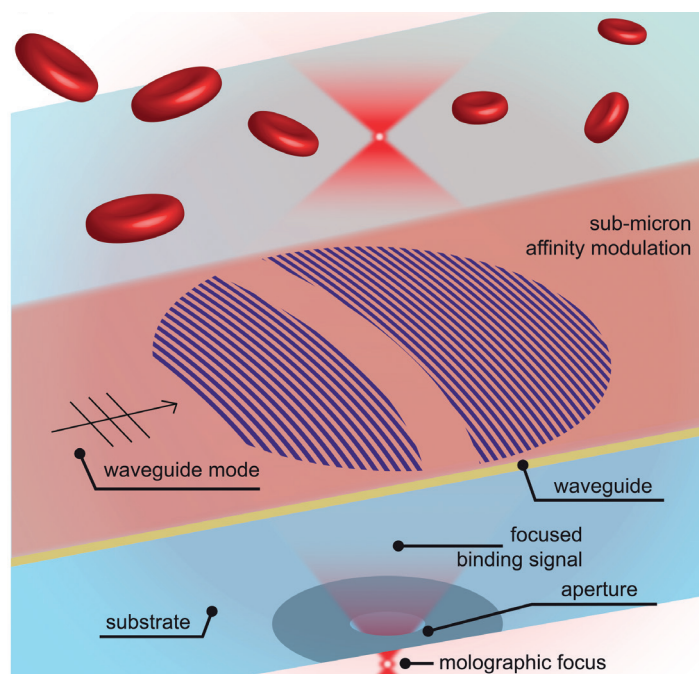


FIG. 1. Focal molography incorporates the essentials of a highly sensitive diffractometric biosensor: A submicrometer affinity modulation formed by specific binders is exposed to a biological sample (e.g., blood). The mode of a high-refractive-index waveguide provides perfect dark-field illumination of the molecules in the vicinity of the sensor surface and enhances the light intensity. The shape of the pattern acts as a diffractive lens, which concentrates the diffracted signal into a focal spot, whereas the background intensity is diluted over the entire solid angle. For efficient spatial filtering, the aperture of the optical system is matched to that of the mologram. (Figure reproduced from [8])

recognition complex is too small to be detected. Only the coherent addition of the individual scattering signals in an ensemble of recognition complexes is detectable.

In principle, label-free optical detection of a specimen of a few pg or less can be achieved by two approaches: One could imagine the specimen to be densely packed as it was the case in Zernike's experiments. This specimen would have a size in the range of one wavelength of visible light. The scattering of light by the dense specimen (i.e. a nanoparticle composed of biomolecules, e.g. proteins or DNA) is visible as a point scatterer under a microscope with dark-field illumination or by phase contrast microscopy. However, a densely packed specimen is completely unsuited for the study of molecular recognition, because molecular recognition requires diffusional accessibility of the binding sites. The second approach is to configure the specimen in a coherent assembly of molecules such that the phases of light scattered by the diffractometric phase object add constructively. A prerequisite for this detection scheme is sufficient coherence of the utilized light source. In a coherent assembly of recognition sites, the sites are spread over distances of many wavelengths (100-1000). Therefore, the recognition sites are sufficiently dispersed that they are accessible to the specimen by diffusion. Thus, only a dispersed (not densely packed) coherent assembly of molecular recognition complexes is suitable for the detection of biomolecular interactions.

A mologram comprises a blueprint of a particular coherent assembly of molecules. Namely, one that arranges the rec-

ognized biological molecules into a focusing diffractometric phase object. The phase object generates a diffraction limited focus from an incident laser beam in close proximity to the chip. The phase object constitutes a "molecular hologram", termed "mologram" in short. A typical diameter of a mologram is 50 μm to 500 μm . A saturated (i.e. fully bound) mologram consists of typically a few pg to 1 ng of bound biological molecules. Therefore, even in the saturated state the biological molecules are only loosely packed and enough open space between the molecules is left. The open space is crucial for the analysis of biomolecular interactions, because (i) molecules can diffuse freely and (ii) the recognition complexes do not influence another to avoid multivalency or avidity effects [12].

2a. Method description: Synthesis of molograms

The synthesis of molograms on a planar optical waveguide on a chip succeeds with properly designed surface chemistry and reactive immersion lithography (RIL) [7]. The RIL process is explained in Figure 2. It allows the creation of the biomolecular recognition structure of the mologram on a light-sensitive non-fouling graft copolymer layer [7]. The synthesis of molograms avoids steric hindrance between the binding sites of the mologram that would occur from overcrowding of molecules in the coherent molographic assembly on the chip [7]. The copolymer layer contains photoactivatable functional groups for patterning of the recognition sites of the mologram under immersion by exposure to light [7]. The RIL process enables tuning of surface chemistries specific to the desired analytical application. It is important

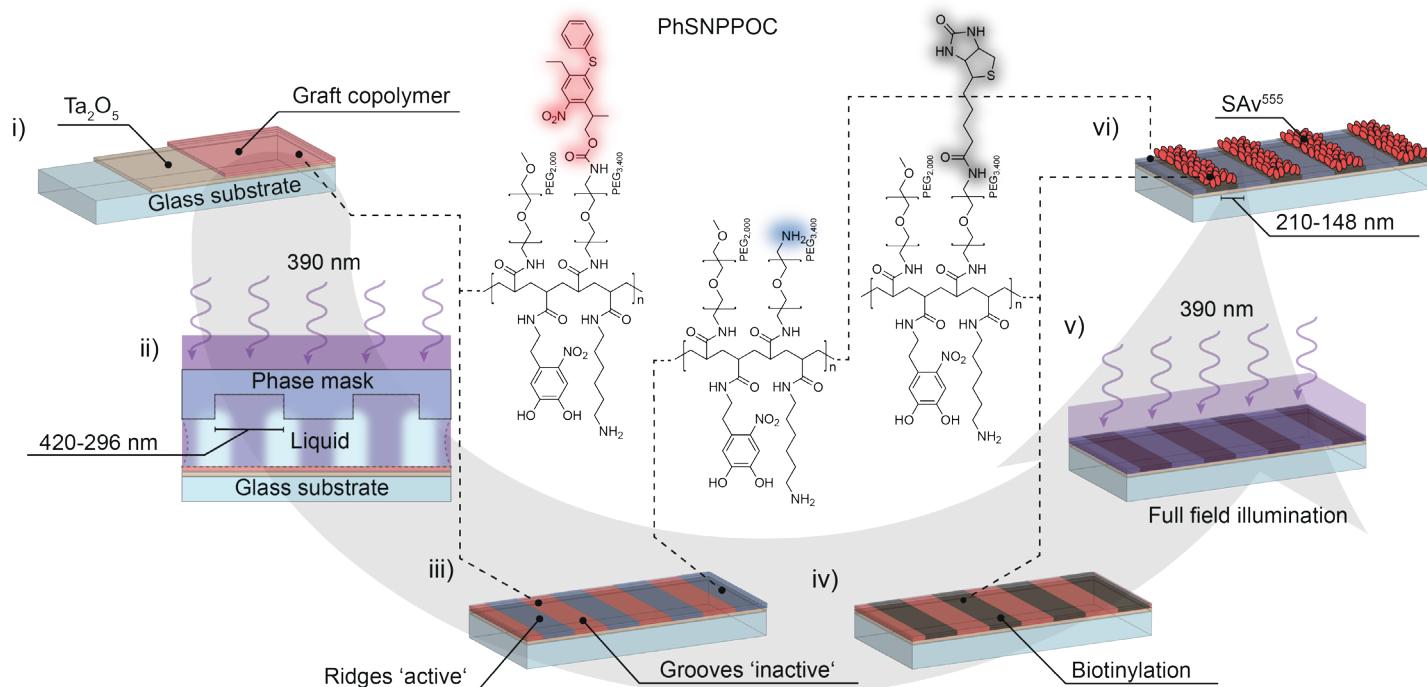


FIG. 2. Schematic illustration of the reactive immersion lithography (RIL) process used in molography, cf. [7]. For a better understanding, the copolymer structures at different stages of the process are connected via dashed lines. (i) The 145 nm metal oxide (Ta_2O_5) waveguide layer is coated with a thin photosensitive graft copolymer layer containing PhSNPPOC protected amino-PEG₃₄₀₀ polymer. (ii) Phase mask technology is used for activation of the monolithic photosensitive layer. The physics of the phase mask lithography process results in structures with half the period of the phase mask (420–296 nm). This allows the creation of the nanostructures (210–148 nm line width) of the mologram. (iii) Induced

activation contrast after photolithography. Activated areas are termed 'ridges' and inactive areas 'grooves'. (iv) Further functionalization with amine reactive compounds leads to the desired binding properties (chemical functionalization). NHS-biotin for streptavidin (SAv) binding. (v) To minimize the difference in nonspecific binding between grooves and ridges, or to realize backfilling of the mologram, the remaining PhSNPPOC groups are photocleaved. Additional passivation (not shown) can be obtained by amine reactive blocking reagents, for example, NHS-PEG. (vi) The molographic signal is generated by binding of SAv. (Figure reproduced from [7])

to note that the used photolithography at a wavelength of 390 nm, at the energy doses used, does not induce refractive index modulations in the copolymer layer [7]. The RIL process was characterized in detail with simulations and stimulated emission and depletion (STED) microscopy [7 and Supplemental Material].

The detection limit of molography can be improved by image reversal reactive immersion lithography [13]. The image reversal RIL process enables the formation of inverse molograms, which exhibit a larger analyte efficiency (i.e. higher quality) compared to standard molograms and therefore require less analyte to achieve a certain molographic signal [13]. As a result, inverse molograms decrease the detection limit of molography whenever the number of molecules available to the sensor is limited. This is the case in most diagnostically relevant applications, which operate either under conditions where the sensor does not equilibrate on a reasonable timescale or in the mass-sensing regime (analyte depletion) [13].

2b. Method description: Read-out of molograms

In this subsection we discuss and exemplify the findings of two publications on molography in journals of the American Physical Society [6, 8].

We imagine a special hologram with three properties: (1) the hologram uses its entire diffractive power for reconstruction of just one image point, a diffraction-limited focal spot that is located in the area of first order diffraction of the incident laser beam. (2) The imagined hologram diffracts a tiny portion of the incident laser beam into its single image point, the diffraction-limited focal spot, and, (3) the hologram is illuminated by evanescent light and its single image point is observed in dark field illumination. In the eyes of a physicist, it is evident that the diffractive power of such a hologram can be extremely weak without loss of its optical function, i.e. the reconstruction of just one first order diffraction focus. The amount of optical material in such a hologram that is required to unambiguously detect a change in the diffracted intensity lies below 10 fg [8]. This is a very small quantity for the detection of a specific molecule in an analytical application. We have shown that a molecular hologram with the described properties can be synthesized [7, 8, 13]. Molography combines the physics of a molecular hologram with the specific recognition between biomolecules [3, 4] to create a small biooptical element for analytical purposes. Dark field illumination of the molograms on a chip is achieved with the evanescent light of a waveguide mode propagating along the surface of the chip [6, 7, 8].

The readout of molograms is based on the analysis of the light in the foci of the molograms [6, 7, 8]. This is achieved in real time by imaging the molographic foci on a photodetector array. The molographic signal in the image plane of a mologram is embedded in a weak background originating from stray light [8]. This background is mainly caused by small non-coherent irregularities in the substrate and the substrate-cover interface. [8]. Due to the random phase and magnitude of the scattered light from the irregularities and non-coherently distributed molecular imperfections the background constitutes a speckle pattern. On the other hand, the diffraction of light by bound molecules at the rec-

ognition sites of the mologram is a coherent process with a defined spatial frequency. In molography, the image plane of the mologram constitutes a Fourier plane and we can therefore employ spatial frequency filtering of this image to separate the molographic signal from the speckle background [8]. The quantification of the molographic signal in this speckle pattern is discussed in detail in reference [8].

From the viewpoint of a physicist, molography can also be understood as a chemical radio [8]. The transmission of radio signals is based on the modulation of an rf carrier signal and the subsequent demodulation at the receiver. Molography applies this principle at optical frequencies to the transmission of chemical signals. Molecules recognize the affinity modulation in the mologram and interact with it. The molecular interaction renders a coherent molecular pattern in the form of a diffractive lens. This diffractive lens modulates the momentum of the guided mode with the spatial frequency of the mologram. The demodulation in k-space is performed by Fourier optics and the molographic signal is separated from the carrier wave in the focal plane of the molographic lens. Molography enables the transmission of chemical signals (e.g. binding information) from coherently ordered molecules in the mologram to a detection point in space (the focus of the mologram).

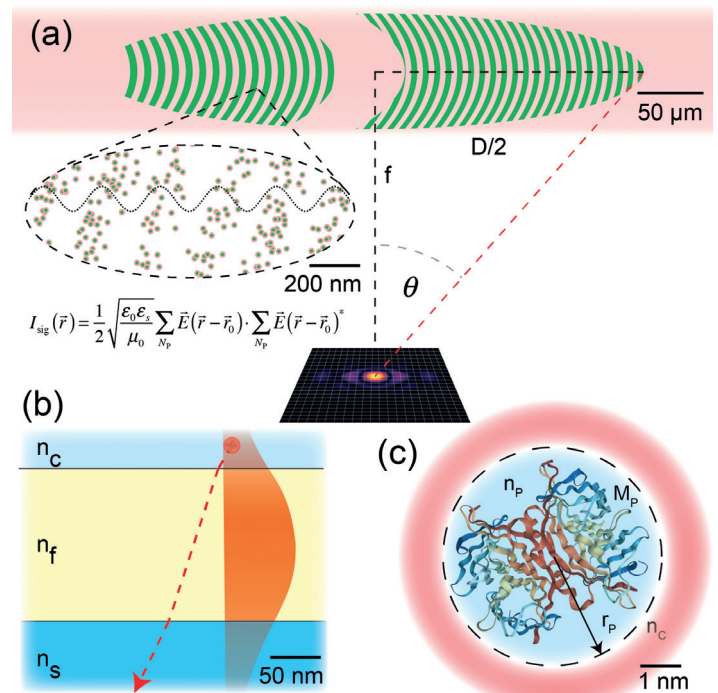


FIG. 3. Simulation of molographic foci, cf. [8]. (a) The molographic signal emerges from the superposition of the scattered electric fields of many individual protein molecules on the surface of the waveguide [proteins are not drawn to scale but their number density corresponds to 2.6 pg/mm²]. This field is computed for every pixel on a specified screen in the focal plane of the mologram. (b) The scattered field is calculated by modeling the proteins as Rayleigh scatterers excited by the evanescent field of the waveguide mode, which is obtained by solving the eigenvalue problem of the slab waveguide. n_c , n_f and n_s are the refractive indices of cover, film, and substrate, respectively. (c) The optical properties necessary to determine the polarizability of the protein dipole, i.e., refractive index and radius, can be calculated from its molecular mass and the refractive-index increment for proteins in water. (Figure reproduced from [8])

3. Applications of molography

Possible applications of molography are vast. They range from the investigation of a specific biomolecular interaction in basic biological research to the diagnosis of a critical health condition in an emergency. To illustrate and to explain the broad applicability of molography we discuss a recently published new method for quantification of molecular interactions in living cells in real time [14]. The method is termed “cell-based molography”. It enables investigations of membrane proteins in their natural environment [14]. The ability to examine biomolecular interactions in living cells in a physiologically relevant context is crucial to the understanding of cellular processes and emanating drug discovery efforts.

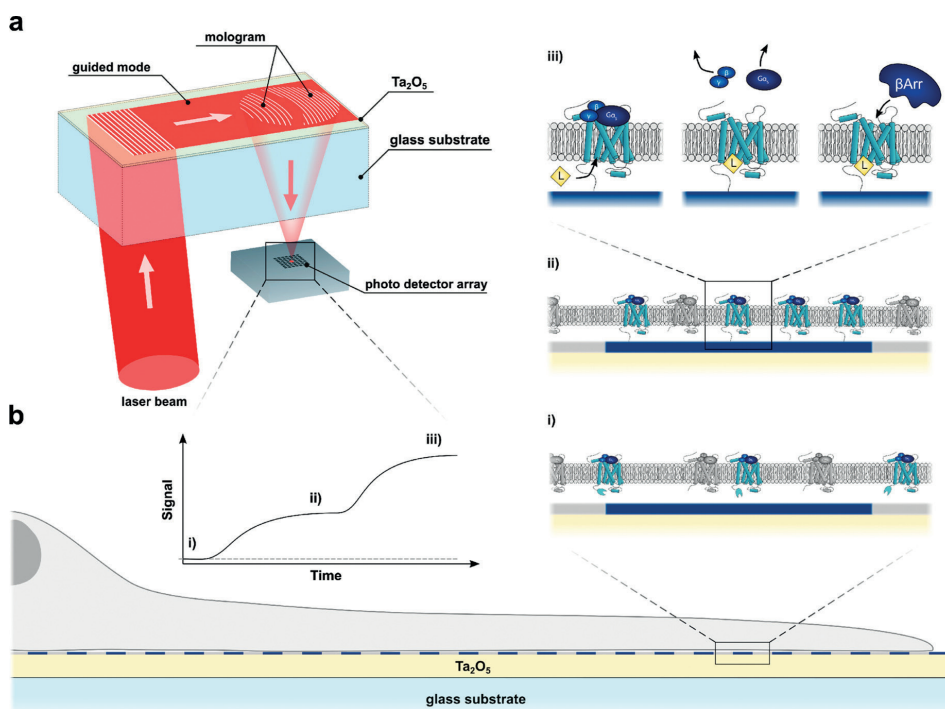


FIG. 4. Illustration of the working principle of cell-based molography, cf. [14]. (a) A single-mode optical waveguide with graft copolymer layer serves as a sensor chip. The guided mode is diffracted at the biomolecules comprising the mologram and forms a diffraction-limited focal spot. Molecules that bind to the mologram contribute to the light intensity in the focal spot, whereas other molecules in the sample do not contribute to this signal. The light intensity scales quadratically with the number of molecules bound to the mologram. The time course of the light intensity is monitored with a photodetector array. (b) Reactive immersion lithography (RIL) is used to generate a template mologram capable of ordering the membrane protein of interest in the adherent cells on the mologram. The autoreactive SNAP-tag protein is fused to the extracellular side of the membrane protein of interest, here a transmembrane spanning, G protein coupled receptor. The SNAP-tag permits one to arrange the target receptor to the template mologram on the sensor chip by covalent binding to the SNAP-tag substrate. Cells are plated onto the sensor chip. (i) Target and off-target proteins expressed in the cells diffuse free-

ly within the plasma membrane. (ii) The randomly distributed target receptors are localized to the mologram on the chip via the extracellular SNAP-tag, leading to a spatial organization of the receptors within the cell membrane. As a result, the mologram is transferred from the surface of the chip into the plasma membrane of the cell, establishing a transmembrane mologram. The number of receptors that are arranged in this fashion can be controlled by the number and therefore the density of SNAP-tag binding sites on the template mologram. Unbound as well as off-target receptors stay randomly distributed. (iii) Refractive index changes at the arranged proteins of interest change the molographic signal. Such refractive index changes are caused either by a local mass change through ligand binding (left), dissociation (middle), or association (right) of cytosolic proteins but also by local changes in ion concentration caused by water or ion influx through a membrane channel. Other molecular interactions, e.g., binding at off-target membrane proteins do not contribute to the molographic signal because they are incoherent. (Figure reproduced from [14])

In cell-based molography [14], cells plated on a molographic sensor chip spread and adhere under standard tissue culture conditions. Thereby the targeted membrane protein in the plasma membrane of the living cells is aligned by a “template mologram” on the surface of the chip. In doing so, the membrane protein molecules transfer the molographic pattern on the chip to the inside of the cell, forming a “transmembrane mologram” (cf. Fig 4b). The transmembrane mologram has the following key characteristics: The coherently arranged membrane protein molecules under study remain in their natural environment but become visible through their coherent arrangement inside the cell membrane. They create a diffraction-limited spot of light, the focus of the transmembrane mologram. Molecules interacting with their intra-

or extracellular domains diffract light into this spot.

Cell-based molography uses a membrane protein nanopattern, the transmembrane mologram within adherent cells, to eliminate disturbing cross-sensitivities in the assay [14]. The required specificity of the molecular detection is achieved by spatially ordering a membrane protein of interest into a coherent pattern of fully functional membrane proteins in the cell membrane on the surface of a sensor chip [14]. Thereby, molecular interactions with the coherently ordered membrane protein become visible in real time, while nonspecific interactions and holistic (i.e. cell shape) changes within the living cell remain invisible [14]. Examples of nonspecific interactions are off-target proteins that interact with non-ordered membrane molecules or the membrane itself as well as other molecules distributed within the cell.

The evanescent field of the guided mode limits the sensitive volume above the surface of the sensor chip to a thin layer of approximately 80 nm thickness. Within this sensitive volume, cell-based molography probes all interactions of molecules with the transmembrane protein in the transmembrane mologram in real time in a living cell.

4. Conclusion and outlook

Thanks to advances in photolithography and nonfouling, photoactivatable surface chemistry, it has become possible to apply the holographic principle to sensitive molecular detection. Such molecular holograms, termed molograms, can be used for analysis of biomolecules in complex biological samples. In molography, biomolecules on a chip become apparent through diffraction, their coherent signal stand-

ing out from the speckle background generated by randomly arranged scatterers. The molographic signal is hardly affected by changes in refractive index due to temperature gradients, buffer changes, or nonspecific bindings, which all are largely incoherent with respect to the molographic pattern.

The processes of life are naturally dynamic in space and time on the intermolecular and the intramolecular level. The amount of biomolecular interactions that may be discovered in the future is huge. Being able to see and to follow biological interactions unfold in time allows us to understand the mechanisms of life as well as disease. Molography allows not only the discovery of biomolecular interactions but also to investigate and to characterize them in complex environments and even within living cells. Molography widens our analytic capabilities for the investigation of biomolecular interactions in a broad range of possible applications.

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Zernike's phase contrast method of observation (1932)

When imaging a biological phase object $u(x,y) = \exp(i\phi(x,y))$ a clearly visible increase in contrast can be achieved either by selective staining of the sample, or more efficiently, by installing a phase plate $A = a \exp(i\alpha)$ in the pupil plane of the microscope objective. The zeroth diffraction-order of the phase object does not carry information about the object. The phase plate should cover only the zeroth diffraction-order of the object-function and should damp it. The damping reduces the amplitude of the zeroth diffraction-order and balances it to

the amplitude of the diffracted waves. The phase plate in the pupil of the objective causes a phase shift between the zeroth order and the higher diffraction-orders of the object-function, which carry the object information. The phase shift α within the object wave leads - after the second Fouriertransformation from the pupil to the image plane - to an intensity variation by the interference term $I(x,y) = C [a^2 \pm 2a\phi(x,y)]$ with C a constant and $\alpha = \pm \pi/2$. Thus, the invisible phase function ϕ in the object plane is observable as intensity contrast variation in the image plane.

(Born & Wolf, "Principles of Optics", Pergamon Press, 1975, p. 424 ff)