Kinetics of Reactive Modules Adds Discriminative Dimensions for Selective Cell Imaging


Dedicated to the memory of Kazuhiko Kinosita, Jr.

Living cells are chemical mixtures of exceptional interest and significance, whose investigation requires the development of powerful analytical tools fulfilling the demanding constraints resulting from their singular features. In particular, multiplexed observation of a large number of molecular targets with high spatiotemporal resolution appears highly desirable. One attractive road to address this analytical challenge relies on engaging the targets in reactions and exploiting the rich kinetic signature of the resulting reactive module, which originates from its topology and its rate constants. This review explores the various facets of this promising strategy. We first emphasize the singularity of the content of a living cell as a chemical mixture and suggest that its multiplexed observation is significant and timely. Then, we show that exploiting the kinetics of analytical processes is relevant to selectively detect a given analyte: upon perturbing the system, the kinetic window associated to response read-out has to be matched with that of the targeted reactive module. Eventually, we introduce the state-of-the-art of cell imaging exploiting protocols based on reaction kinetics and draw some promising perspectives.

1. Living Cells are Singular Chemical Mixtures to Analyze

With respect to current mixtures found in the field of analytical chemistry, living cells exhibit several features making them highly singular: 1) Transcriptomics,[1–3] proteomics,[4–6] metabolomics approaches[7–9]—sometimes carried out down to the single cell level[1,5,6,13]—have shown that cells contain an extremely large number of components (typically more than 10⁶), which essentially share similar elemental composition and functional groups; 2) The molar concentrations of the cell components cover an extremely large dynamic range, from 10⁻¹⁰ mol L⁻¹ for ions such as Na⁺, K⁺, or Cl⁻, to 10⁻¹² mol L⁻¹ for DNA,[12] 3) Cells exhibit a multi-scale spatial heterogeneity and a multi-scale dynamics at steady-state.[16] Their components are heterogeneously but precisely distributed in multiple compartments. Furthermore, whereas the genome is more or less static, the transcriptome and the proteome evolve on a time scale of minutes to hours[11] while the metabolome fluctuates on the second time scale;[12] 4) Cells experience an out-of-equilibrium living state.

Understanding the inner workings of cells, which are regulated by complex processes involving a large number of genes, necessitates the measurement of various aspects of their phenotype.[13] The challenge then resides in obtaining multiplexed images of living cells reporting on a large number of distinct molecular species. Addressing a given component in a mixture without any separation generally requires a label that contains either specific atoms or functional groups and which is observed by analyzing its interaction with an interrogating tool (e.g. light). Yet, the endogenous labels borne by cell compo-
ponents are often not different enough to permit discrimination of a given target among the myriad of similar biomolecules. To circumvent such limitations exogenous labels\(^1\) (e.g. fluorescent dyes conjugated or not to reporting reagents) are widely used to render a given target unique.\(^{14,15}\)

Since the number of molecular readouts that can be measured in single cells remains limited in most cases to a handful of species (vide infra), multiplexed detection requires specific strategies. Up to date, the most impressive achievements have used oligonucleotide- or antibody-based probes reacting with their complementary targets. Barcoding of reporting reagents with combinations of different fluorophores has allowed simultaneous imaging of up to 32 targets in super-resolution microscopy.\(^{17}\) Alternatively, iterative staining followed by stain removal procedures in which the successive reagents are conjugated to the same dye have given access to 50–100 molecular readouts.\(^{18–21}\) However, these approaches are limited to the study of fixed, permeabilized cells.

Another relevant question remains whether a high extent of multiplexing in living cells is even possible. For a long time the answer has been negative. Indeed, the number of molecular species that could be studied simultaneously was limited by our ability to genetically label more than three or four of them with distinct reporting tags (e.g. fluorescent proteins). However, this situation has recently changed with the development of powerful tools enabling targeted genome editing (e.g. CRISPR-Cas9 system).\(^{22}\) Combining these new techniques with innovative imaging strategies, such as those discussed in this review, should now make possible live monitoring of a large number of different targets.

2. Why and How to Use the Kinetics of Reactions for Selective Imaging?

Considering that limitations related to labeling should soon be solved, the challenge is now to increase the discriminative power of the microscopies used to read the labels. Indeed, most of the reported protocols are based on the acquisition of a single observable (e.g. the absorbance, the fluorescence intensity) from the target or its reporting reagent and, even in the most favorable cases, one faces spectral crowding (vide infra). Furthermore, scattered light and autofluorescence are inherent to cell imaging and often contaminate the useful signal. Upon adopting both a conceptual and a practical point of view, this section explains how the kinetics of reactions in which the labels are engaged can fruitfully be exploited for selective contrast generation.

2.1. Discriminative Strategies

Any analytical protocol involves processes that can be modelled as a reactive module, that is, a set of reactions joining nodes corresponding to reactants and/or products (Figure 1). For instance, observables such as absorbance or fluorescence intensity originate from realizing the forward and the backward transition between the ground state and the first singlet excited state (Figure 1a), whereas a titration exploits the chem-

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1 Unambiguous identification of native analytes can be achieved by emphasizing physicochemical criteria (e.g. separation followed by analysis by mass spectrometry).\(^{16}\) However, such invasive methods are out of the scope of the present article.

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Figure 1. Examples of reactive modules involved in analytical strategies. a) Exchange between the ground and the first singlet excited states of a fluorophore. The absorption and fluorescence emission are up and down conversion processes, respectively. They are linked to the absorbance and fluorescence intensity observables, which are informative of the fluxes of the associated processes; b) Titration involving the reaction of a specific reagent \(\mathcal{R}\) with a desired analyte \(\mathcal{R}\) to yield a product \(\mathcal{P}\); c) Mechanisms accounting for the behaviour of a photoswitchable label upon illumination. Light absorption drives the exchange between the two ground states of the label, denoted 1 and 2 (in the considered scheme, state 1 is supposed to be thermodynamically more stable than state 2). Mechanistically, this exchange involves light absorption leading each ground state i (i = 1 or 2) to its excited state \(i^{*}\), which subsequently relaxes either by giving back the i state (for instance by emission of a photon when the probe is a photoswitchable fluorophore) or by photoisomerization (yielding the other ground state). The ground state 2 can also notably relax thermally toward the more stable state 1. Beyond a time scale of a few tens of nanoseconds, the latter multi-step scheme dynamically reduces to a two-state exchange between a reactant \(\mathcal{R}\) and a product \(\mathcal{P}\) (adapted from Ref. [25]). In b and c, the observable is the extent of a reaction (the titration reaction or the reduced two-state exchange), which is accessed by measuring the relative proportions of the reactant and product states by means of another probing process (e.g. light absorption or fluorescence emission). More generally, any analytical strategy may be considered to rely on a process, which can be modelled as a reactive module involving reactions (characterized by rate constants) and nodes (identified to reactants and products).
change is further characterized by the ratio $K_{ij}(t) = k_{ij}(t)/k_{ji}(t)$, which identifies with a thermodynamic constant at chemical equilibrium, and by $\tau_{ij}(t) = 1/(k_{ij}(t) + k_{ji}(t))$, which provides the relaxation time of the process.\cite{23} In fact, this simple two-state exchange is sufficient to accurately model many reactive modules. As a first example, it is appropriate to dynamically model a titration reaction when the analyte is present at concentrations higher than the reporting reagent (Figure 1b).\cite{24} It also results from dynamically reducing at a large enough time scale the multistep reactive module involved in a photoisomerization (Figure 1c).\cite{25}

In a complex mixture, a first level of discrimination against interfering species can be achieved by restricting the realization of a two-state exchange to the targeted component. For instance, in absorbance and fluorescence emission measurements the observable is sensitive to the energy associated with the two-state exchange so as to permit spectral discrimination. In contrast, titration relying on biosensors will benefit from the high selectivity of molecular recognition in biology.\cite{26}

A second level of discrimination can result from singularizing the response of the targeted analyte to the two-state exchange [Reaction (1)]. As an example, in the case of a titration it will consist in adopting a reporting reagent which gives the largest extent of reaction with the targeted mixture component. In most cases, the reactive module is read out at chemical equilibrium or at the steady state. Under such conditions, the rate constants $k_{ij}(t)$ and $k_{ji}(t)$, and the ratio $K_{ij}(t)$ adopt constant values $k_{ij}^0$, $k_{ji}^0$, and $K_{ij}^0$.\cite{27} The concentrations in states 1 and 2 as well as the fluxes of the forward and backward reactions are then constant. The single associated observable, $O^*$, is correspondingly constant and generating a high enough contrast with interfering signals relies on optimizing its value only.

Protocols exploiting perturbations of a control parameter $P$ are expected to be much more discriminative. Indeed, when the system is submitted to a temporal perturbation $P(t)$, its rate constants $k_{ij}(t)$ and $k_{ji}(t)$ as well as the concentrations in states 1 and 2 may evolve in time so as to generate not anymore one observable but a temporal series of observable values $O(t)$. The latter series depends on $P(t)$, which is common to all the mixture components, but it also depends on the impact of the perturbation on the two-state exchange through the variations of the two rate constants. It thus brings a kinetic dimension to the discriminative approaches relevant at equilibrium or in a non-equilibrium steady-state. In particular, in addition to the extent of the label response to the perturbation, one can exploit the difference of relaxation times between the targeted reactive module and the interfering species to filter the overall system response and selectively retrieve the signal issued from the desired analyte (Figure 2).

**Figure 2.** Principle of cell imaging under kinetic control. a) When all control parameters $P$ are constant, the discriminative reactive module can be observed at steady-state or chemical equilibrium (depending on the considered case). The single associated observable reporting on the labeled cell components, $O^*$, is constant and may be not enough discriminative. Thus possible misinterpretations can be introduced in the resulting “standard” image when the number of distinct labels is too large. The target (here the reactive square) cannot be reliably distinguished; b) in contrast, observation performed upon perturbing the system by applying a temporal modulation of a control parameter $P(t)$ generates a temporal series of observables, $O(t)$. When the kinetic windows associated with the response of the targeted analyte and with the response reading-out are matched, the contribution of the targeted label can be selectively extracted by demodulation from the overall response to provide a kinetically filtered image essentially revealing the distribution of the target only.

Selective imaging exploiting the kinetics of reactive modules is the focus of the present review. However, here it is worth to mention that it shares several features with both dynamic contrast imaging and modulation spectroscopy, whose implementation also enables one to discriminate between a given compound and interfering species in the frequency domain.\cite{28} In dynamic contrast imaging,\cite{29} the labels are subjected to a periodic excitation to which only they respond and a demodulation scheme is applied to selectively extract their signal from a submerging background. Periodic excitation here generally relies on physical phenomena (e.g. nanoparticle rotation\cite{30–32} or spin state manipulation with a magnetic field,\cite{33,34} thermal lensing\cite{35–37} or surface reflectance alteration upon IR heating).\cite{38,39} Moreover, the detection scheme does not exploit the dynamics of the label response to achieve discrimination but mostly takes advantage of synchronous detection to improve the signal-to-noise ratio.\cite{40} On the other hand, modulation excitation spectroscopy, a special case of 2D correlation spectroscopy,\cite{41} is applied to overcome the limitations of spectral crowding in mixtures and to level off the significance of intense background as well as noise.\cite{42,43} Periodic excitation of an ensemble of reactive species leads to periodic concentration changes for

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2 Throughout the Review, relaxation times associated with reactive modules are denoted $\tau$, whereas characteristic times related to the instrumentation are written $t$.

3 The $0$ in the exponent refers to a situation where each control parameter $P$ is constant. Later on, the $1$ in exponent will indicate that we consider the first-order expansion with respect to a small perturbation in $P$. Details on the possible control parameters are given in Section 2.2.1.

4 A definition of this term is provided in Section 2.2.3.

5 We only found a few papers demonstrating that, similarly to what we presently discuss for the field of chemistry, it is possible to utilize the difference in response time of physical phenomena to generate contrast.\cite{44,45}
which phase lags depend on the mixture components. A phase-sensitive detection is used to demodulate the whole system response and it yields a two-dimensional spectrum in which a phase dimension related to chemical kinetics adds to the original wavelength dimension classically observed in the absence of periodic excitation. Interestingly, matching the radial frequency of the control parameter modulation with the inverse of the relaxation time associated with a given reagent permits the selective extraction of its spectrum.

2.2. Practical Implementation

The conceptual framework underlying the present kinetic discrimination strategies is actually that of relaxation methods. This group of techniques has been extensively used to provide rate constants and mechanisms in chemical kinetics: the equilibrium state of a reactive system is perturbed and information on its kinetics is retrieved from how the reaction extent evolves in time to satisfy the newly imposed experimental conditions. In line with classical accounts and more recent contributions on related topics, this section begins with the choice of the perturbation parameter, then it deals with the selection of an observable that reports on the reaction extent, and it ends up with the various protocols of signal acquisition and data processing harnessed to generate the kinetically filtered image.

2.2.1. The Perturbation Parameter

A first means to bring a reactive system out of equilibrium consists in changing the values of the equilibrium and rate constants by acting on a physical parameter, for instance temperature or pressure. Modifying the amount of interacting entities represents a second method to perturb an equilibrium state and, in practice, one may vary either species concentrations (in the case of chemical reactions) or the photon flux (in the case of light-driven transitions). In this subsection, we shortly review the four perturbation parameters with respect to their use for reaction rate measurement and kinetic discrimination.

Temperature

Most biologically relevant processes exhibit an enthalpy of reaction typically lying in the 20 to 200 kJ mol\(^{-1}\) range,\(^6\) which results in at least one significant activation enthalpy. Consequently, a change of a few Kelvins can often alter both equilibrium and rate constants in a measurable way, while remaining in a regime where rate equations can be linearized. Temperature has thus been widely used to investigate mechanisms and determine rate constants.\(^{23,42,43,51–53}\) Its variations can be implemented in many manners. Sudden jumps have been obtained by the Joule effect subsequent to capacitor discharge in the buffer,\(^{31,54}\) by IR laser absorption by the solvent, or by thermal transfer from a fluid flowing at a different temperature. All the associated time-domain measurements have been followed by UV/Vis, IR, circular dichroism, and fluorescence spectroscopies. The accessible temporal range depends on the heating technique, it runs from a few picoseconds when relying on water vibrational relaxation subsequent to IR absorption to several tenths of seconds when heat has to diffuse over hundreds of micrometers.\(^{56}\) Alternatively, temperature oscillations can be produced using a thin-film resistor or an IR laser and the response of reactive biomolecules between a few milliseconds to a second can be acquired thanks to calorimetric or fluorescence detection. In addition, theoretical investigations have suggested that the frequency domain could favor the development of protocols able to validate complex mechanistic schemes.\(^{69,70,71}\)

Temperature changes of small amplitude are non-invasive and compatible with cell imaging. Furthermore, varying this parameter according to any arbitrary temporal profile is becoming easier since, in addition to IR laser heating, it is now possible to rely on emerging technologies related to microsystems and nanoparticles. It is here worth noting that, in the two latter cases, thermalization relies on heat diffusion and thus the best accessible temporal resolution, \(t_{\text{diff}}\), greatly depends on the size of the heated volume, varying from \(10^{-5}\) s for the surrounding of submicrometer-size gold colloids to \(10^{-3}\) s for microfluidic observation cells. Eventually, numerous fluorescent molecular thermometers able to map temperature in vivo have already been devised, offering opportunities for a better control of the excitation signals.

Although only proof-of-concept experiments on artificial model systems have been presently realized, the recent determination of chemical relaxation times in a cell\(^{66,74}\) suggests that thermal perturbations for selective imaging should soon be implemented in living systems.

Pressure

Reactions involving biomolecules most often display reaction volumes between 10 and 100 mL mol\(^{-1}\). Therefore, pressure jumps of up to hundreds of MPa have to be used to significantly shift the extent of chemical reactions and enable one to determine rate constants.\(^{23,42,43}\) Various minimal relaxation times can be accessed, from the millisecond for instruments relying on valves and transducers to the microsecond.

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\(^6\) Although jumps or oscillations of electric field have been historically important in the development of relaxation methods, they will not be considered here since their scope for cell imaging seems rather limited. Similarly, we will not report on the use of magnetic field because up to now it has been restricted to the detection of very specific labels (e.g. bimolecular electron donor–electron acceptor pairs, nitrogen-vacancy centers in nanodiamonds).\(^{23,42}\)

\(^7\) The values of thermal diffusivity in PDMS, water, glass, and silicon are respectively 0.16, 0.14, 0.81, and 76 \(\times 10^{-4}\) m\(^2\) s\(^{-1}\), which for a distance of 100 \(\mu\)m yields characteristic times of about 62, 71, 12, and 0.16 ms.

\(^8\) The subscript \(P\) here refers to the perturbation, as it will be the case with \(O\) for the observable, and \(F\) for the function used to compute the kinetically filtered data. The superscripts min and max indicates that we are dealing with instrumental limits.
for those based on membrane rupture.[22,105,106] The speed and the attenuation of ultrasonic waves have also been measured to investigate the dynamics of reactive systems in the nano- to the microsecond time range.[23,42,107,108] Although these methods have been extensively used to study protein folding[100,101,106,108] and ligand binding[103,104] the required large pressure changes seem incompatible with cell culture. Furthermore, the rather severe experimental constraints would surely make difficult the implementation of the associated experimental setups on an optical microscope.

Concentration

Rapid mixing of reagents is certainly the most popular principle to retrieve kinetic information. It can be implemented in bulk using a stopped-flow apparatus or some other rapid mixing devices,[42–44,109] or it can be achieved at the surface of a solid-state sensor.[110,111] Usually one analyzes the response to a concentration step in the time domain. However, technical developments based on concentration oscillations and frequency-domain data processing have also been reported.[112,113]

Most commercial setups involve significant shear flow and measurements at the cell surface of association kinetics faster than the second have been very scarce[114–117] until microfluidics became common use.[118,119] Upon relying on miniaturized devices, it is now easier and less invasive to generate controlled jumps[120,121] or oscillations[122–125] of concentration in signaling molecule around adherent cells, which enables one to unravel the complex dynamics of regulation pathways.

Despite these many advances, accessing the cell interior to modify a concentration remains difficult if the reagent is not permeant to the cytoplasmic membrane. A first solution utilizes a micropipette to inject the desired molecule.[126] Nevertheless, such a tool exhibits a limited temporal resolution (a few seconds) and implies convective flows which may be detrimental to living objects. An alternative makes use of a focused wave, which locally decomposes the targeted compound or releases it by activation of a precursor. The diversity of triggers has recently been expanded to ultrasounds[127] and X-rays[128] but UV/Vis light remains the most popular, both for decomposition[129–131] and activation.[119,132–142] In the latter case, a significant issue is the quantification of the delivery. First, calibration of the incoming photon flux can be difficult because of the uncertainties associated with an optically heterogeneous medium such as a cell. Second, the local concentration in the precursor may be unknown because of some uneven repartition between the different subcellular compartments. One possible way to address this quantification problem relies on the simultaneous release of a reporter in a one-to-one molar ratio with the molecule of interest.[143–148] Eventually, one should here mention that loading the cell with magnetic nanoparticles functionnalized with a given molecular species can be used for local enrichments or depletions, by applying appropriate magnetic field gradients.[149–151]

The minimal accessible time for perturbing concentrations, $t_p^{\text{min}}$, generally depends on the mixing rate. Although they would yield faster mixing than by diffusion, shear flows would be often detrimental to living cells. In contrast, molecular diffusion is harmless and this transport process is usually favored to generate concentration changes in the close surrounding of biological objects, typically in the few tens of microns. As a consequence temporal resolution is rather low, with $t_p^{\text{min}}$ ranging between 1 and 100 s.[119] In the specific case of triggered mediated activation of a precursor, the relaxation time of the activation process has also to be taken into account for determining $t_p^{\text{min}}$.[142] Hence, when light is used, photo-induced liberation of a target (uncaging) should typically occur after $10^{-9}$–1 s depending on the caging group.[119,132–142] Such a temporal resolution is much below the one of the light sources (vide infra). Moreover, since uncaging often involves thermally-driven reactions, spatial resolution may be as well decreased.[155,156]

Light

Illumination intensity is a parameter of choice to modify the value of the rate constants in the particular case of reactive schemes involving a photoactivation step. Variations of photon flux have thus been extensively used to characterize the dynamics of photochemical[157–159] and photophysical[160–162] processes. Experimental investigations in cuvettes are numerous and their presentation is out of the scope of this review. They have been conducted in both the time and the frequency domain, on synthetic and biological species. As far as the microscopy of living samples is concerned, commercial setups are now available to map the lifetime of the excited state of luminescent molecules.[153–160]

In fact, light exhibits several attractive features as a control parameter for implementing imaging protocols based on kinetic discrimination in life sciences. Moderate photon fluxes are non-invasive, as evidenced by the numerous optical microscopies developed to observe living cells or organisms. They can be delivered with an excellent spatial resolution: a light beam can be focalized down to the diffraction limit, which permits to confine photoactivation to a few femtoliters—three orders of magnitude smaller than a cell—when relying on multiphoton excitation. Similarly, the associated temporal resolution can be very good, down to the femtosecond range when using a pulsed laser.[160] Varying the illumination intensity can also easily be achieved with acousto-optic modulators, shutters, or more simply LED sources (giving access to $t_p^{\text{min}}$ values above 0.1 μs). As a consequence of such favorable attributes, much of the dynamic imaging techniques established so far are based on photocontrol.

2.2.2. The Observable

The observable used for reading out the discriminating reactive module contributes to specify several key features of an...
Although Part of the light (o’2016 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim cm cm cm nm nm nm Hence, to absorb as ignificant amount of incident pho-

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m m 1) An pump beam is absorbed by the label, which

nm range, For a g old nano-

m m m In brightfield light microscopy, a cell can generate contrast

either by scattering or by absorbing some of the incident pho-

tons. Even if light scattering can be evidenced in phase con-

trast microscopy, cellular components exhibit refractive indexes
close to the one of water so as to inherently yield low contrast images. In principle, light absorption would be more favorable since chromophores are endogenously present in biological media and since it is also possible to introduce exogenous labels. Nevertheless, matter remains quite transparent and di-

rectly evidencing light absorption is highly demanding at the

single cell level.

In the UV/Visible wavelength range that drives transitions

both between electronic levels, the atoms of even the best available organic (e.g. cyanines) and inorganic (e.g. gold nanoparticles) chromophores exhibit ratios between their light absorption cross section and their physical cross section lower than 10%. Hence, to absorb a significant amount of incident pho-

tons over a light pathway of ~10 μm, which is the typical di-
meter of a cell, would require labels to be present at 0.1 to

1 nm. In fact, most endogenous as well as exogenous chroomophores possess lower cross sections for light absorption and are present at submillimolar concentrations. Thereby, only a marginal part of the incident light is expected to be ab-

sorbed at the single cell level. Furthermore, since one deals

with small differences between large numbers, it should also

be highly difficult to discriminate between light absorption

and phenomena such as light reflection and scattering.

In the infrared range, the spectral domain associated with

transitions between vibrational levels, the cross sections for

light absorption are typically three to four orders of magnitude lower than the ones characterizing the transitions between electronic levels. It thus restricts the use of the corresponding functional groups to image the most abundant components of cells. In addition, recovering specific signals in the IR range is made difficult by the strong absorption originating from water. Despite such drawbacks, there is a recent effort to develop exogenous labels displaying large absorption coefficient in a wavelength range where no endogenous chromophore nor water would absorb.

To overcome the preceding limitations, approaches in which

optical absorption is indirectly detected have been developed. One here does not anymore measure the ratio between the amounts of light impinging on the sample and transmitted through it but relies on physical effects following photon ab-

sorption. The acquired signals are now essentially proportional to the quantity of absorbed light, even in the presence of re-

fections or scattering.

Photothermal microscopy relies on two-color illumina-

1) A pump beam is absorbed by the label, which

causes heat release and yields a change of local refractive index; 2) a probe beam has its propagation modified by the thermal lens. Its wavelength is chosen outside of the absorp-

tion range of the label so that its power can be increased to very high values, for which photon noise may become extremly small. Consequently, noble metal nanoparticles, quantum dots, or single-walled carbon nanotubes have been suc-

cessfully observed down to the single-object level.

Photoacoustic microscopy combines optical illumination and ultrasonic detection with transducer arrays. Part of the light energy absorbed by the label is released as heat, leading to a transient thermoelastic expansion and correspondingly to the propagation of MHz sound waves, which are then analyzed according to their arrival time to produce images down to the single cell-level. Interestingly, there is presently an effort to develop specifically dedicated genetically-encoded probes for such a microscopy.

Both the sensitivity of absorption bands to the environment (e.g. solvent polarity, electrical fields, pH) and the ratio of their width at half height over the whole spectral window govern the capability to selectively address a targeted chromophore by matching the wavelength of the incident light with the one of its absorption. In fact, absorption spectra are often signifi-

cantly medium-dependent. Moreover, in condensed phases and for transitions both between electronic and vibra-

tional levels, the ratio evaluating the relative spectral band-

width remains rather large: it lies in the 5–10% range at best. Hence, absorption spectra are often subjected to spectral

10 Strongly absorbing organic chromophores exhibit molar absorption coeffi-

11 ci ents in the 10^-5 m^-1 cm^-1 range. which correspond to optical cross sec-

tions α ~ 4 x 10^-11 nm^2, and their surface is the nm^2 range. For a gold nano-
sphere of 40 nm-diameter, the molar absorption coefficient is

7.7 x 10^-16 m^-1 at 530 nm. Thus its optical cross section

(α = 3 x 10^-10 nm^2) fairly compares to its physical cross section (1.5 x 10^-10 nm^2). In contrast, the optical cross section of one of its gold atom is about

α = 0.15 x 10^-10 nm^2 whereas the physical cross section of a gold atom is about 6.1 x 10^-16 nm^2.

12 Such numbers could be made more favorable by relying on cavity-ring-down spectroscopy (CRDS), in which one measures the rate at which light from a pulsed laser source leaks out of a stable, high-finesse, optical cavity formed by two highly reflecting mirrors. Minimum detectable absorp-

tion down to 10^-8 cm^-1 has been reported, which is equivalent to picomolar concentrations for strong absorbers.
crowding in the case of mixtures, which makes difficult the implementation of multiplexed observations.

Fluorescence

Fluorescence emission is a radiative deexcitation process following photon absorption: the fluorophore relaxes back from its first excited electronic state to its fundamental one upon emitting a photon. The associated observables are the fluorescence intensity as well as the lifetime of the first excited state (typically in the nanosecond range for bright fluorophores)\(^{[162]}\).

Since only a few species significantly fluoresce, microscopies based on this observable are usually highly selective. Moreover, the excitation light can easily be rejected from the useful signal because they are spectrally separated by the Stokes shift. The emitted photons are thus detected over a low background, a feature that can further be improved by increasing the spatial confinement of the excitation volume.\(^{[1]}\) As a consequence, fluorescence techniques are highly sensitive, down to the single-molecule level.\(^{[194–196]}\)

Such favorable features have led fluorescence to become the most universally adopted observable for sensitive and non-invasive imaging of living cells. However, very few metabolites and biomolecules are natively fluorescent, which necessitates to rely on exogenous labels.\(^{[14, 15, 197]}\) In addition, the technique suffers from several limitations: 1) light scattering as well as autofluorescence from endogenous fluorophores such as tryptophan (emitting in UV wavelength range) and retinol, flavins, or riboflavins (emitting in the visible spectrum) can interfere with the signal of interest; 2) emission bands are rather broad, typically beyond 50 nm. As for light absorption, one faces spectral crowding and only a few biomolecules can be simultaneously imaged;\(^{[198]}\) 3) fluorophores suffer from fatigue after a variable number of excitation-deexcitation cycles and irreversibly bleach.

Before closing this subsection dealing with spontaneous fluorescence emission, it is worth mentioning that stimulated fluorescence emission has been also recently explored:\(^{[200]}\) relying on a modulated stimulating beam, it has been used to image the absorption of a few tens of molecules in cells. Beyond expanding the scope of fluorescence microscopy to non spontaneously fluorescent chromophores, this approach could interestingly limit photobleaching since stimulated emission could be generated before competing detrimental processes would occur.

Raman Scattering

Devoid of any hampering originating from water absorption, Raman scattering is more relevant than IR absorption to image transitions between vibrational levels in biological media. Hence, Raman microscopy has emerged as a non-invasive technique, that is, free of any labeling step, able to trace abundant species in living cells. However, since acquisition speed and sensitivity are limited (image acquisition typically takes 1–100 s depending on the signal strength),\(^{[115]}\) coherent anti-Stokes Raman scattering (CARS)\(^{[201, 202]}\) and stimulated Raman scattering (SRS)\(^{[203, 204]}\) have been recently introduced as powerful alternatives. In CARS and SRS the vibrational frequency of the targeted bond is excited by means of the beating between two spectrally shifted ultra-short (ps or fs) laser beams, instead of a single continuous wave like in spontaneous Raman. As a result, the Raman signature is significantly enhanced by more than five orders of magnitude. Although powerful to perform vibrational imaging, CARS is impeded by a non resonant background and a nonlinear dependency with molecular concentration. This is not the case of SRS that is linear with concentration and has a much reduced background\(^{[205]}\) opening new routes for chemical imaging in biological media. In particular, SRS has been shown to permit live-cell imaging of small biomolecules bearing alkyl label.\(^{[206]}\)

2.2.3. The Generation of the Kinetic Contrast

Relaxation methods have classically been divided into transient and stationary ones.\(^ {\text{[21, 42]}}\) In the first category, mechanisms and rate constants are obtained from analyzing the observable time trace \(O(t)\) after a sudden pulse or jump of the control parameter \(P\) (Figure 3 a). In the second category, the excitation function \(P(t)\) is oscillatory and one measures the amplitude \(O'(\omega_0)\) and the phase \(q'(\omega_0)\) of the harmonic response of the reactive system at various radial frequencies \(\omega_0\) (Figure 3 b). Fitting the two latter curves then yields the same kinetic information as the one obtained in the time domain. Indeed the two functions, \(O(t)\) and \(O'(\omega_0)\), are Fourier transforms of each other\(^ {\text{[13]}}\) and thus possess the same information content\(^ {\text{[21, 42]}}\). Despite these formal similarities, we show below that selecting one or the other protocol bears significant experimental implications.

[^12]: Single-plane illumination microscopy (SPIM),\(^{[140]}\) which confines excitation to the observed plane by lateral illumination with a light sheet is a representative example. Total internal reflection fluorescence (TIRF) microscopy\(^{[199]}\) provides another relevant avenue restricting excitation to the close neighborhood of a surface by relying on illumination by an evanescent wave. Independently, some other advances in fluorescence imaging are worth mentioning. Although spatial resolution in optical microscopy is generally limited by the diffraction limit at the considered wavelength (typically around 250–300 nm),\(^{[202]}\) several super-resolution techniques have recently been developed to lower this limit down to a few tens of nanometers.\(^{[194–196]}\)

[^13]: Experimental signals are polluted by noise, whose amplitude has a general tendency to increase at low frequency. Therefore, although the analysis in the real space (i.e. in the time domain) and in the Fourier space (i.e. in the frequency domain) leads to the same basic information, it is more difficult to separate the signal from the noise in the first case than in the the second one. Two reasons explain this issue: 1) When a Heaviside function is applied to the system, the response is an exponential decay which involves all the low frequency components; 2) In contrast, the periodic modulation used for Fourier analysis is directly an eigenfunction of the differential equation and it is completely localized in Fourier space. By averaging over several periods, one escapes the low frequency perturbation and directly improves the signal-to-noise ratio.
In order to adjust originating from interfering, the time constant of the acquisition setup, is much smaller than the relaxation time over.

In this specific case, the sine demodulation varies as the square of the frequency.

Since most of the emitted photons are usually discarded, repetitive acquisition cycles may be necessary, in particular to selectively image reactive systems with very small \( \tau_r \).

**Frequency-Domain Imaging**

In this second approach, the perturbation function \( P(t) \) is periodic. Generally it is a sine function “\( P(t) = \sin(\omega_F t) \)” but without any absolute necessity. As in the time-domain strategy, the characteristic cutoff frequency of the perturbative instrument, \( \omega_F^{\max} \approx 1/\tau_p^{\min} \), is significant upon constraining the amplitude of the control parameter modulation that can be reached at a given excitation frequency \( \omega_p \). The periodic modulation of \( P \) drives the reactive modules to also adopt a periodic behavior and the temporal evolution of the observable is employed to produce a filtered image by demodulation. More specifically, for each pixel of the sensor, one computes \( \varphi = \int_0^\tau \sin(\omega_F t + \varphi_F) dt \) where \( \omega_F \) and \( \varphi_F \) are the demodulation frequency and phase, respectively, and \( n \) is the number of periods over which the operation is performed (Figure 3b). This \( \varphi \) expression is useful to introduce the different protocols that can be found in the literature. For instance, collecting the Fourier transform of \( O(t) \) implies varying \( \omega_F \) over the whole accessible range while taking \( \varphi_F \) equal to 0 or \( \pi/2 \).

Yet, demodulation is often performed at the single frequency \( \omega_F = \omega_p \) according to a protocol called homodyne detection. In this specific case, the sine demodulation function is sometimes replaced by another periodic function having the same fundamental frequency as the one of the stimulus. Furthermore, the technique becomes known as cross-correlation, when one relies on some signal issued from the sample itself to demodulate the time trajectories of the various pixels. Performing an auto-correlation analysis is even possible for reactive system whose observable switches between two colors.

All the above demodulation techniques share a common feature: they are narrow band, which means that all the Fourier components of \( O(t) \) that are not at radial frequency \( \omega_F \) will be rejected. In particular, it includes the background signal arising from the species that do not oscillate with the excitation, as well as most of the noise. Indeed, such acquisition schemes are much similar to lock-in detection. Nevertheless, if several reactive modules respond to the perturbation, the contribution of the targeted one still needs to be enhanced with respect to the interfering ones. This goal can be achieved by tuning both \( \omega_F = \omega_p \) and \( \varphi_F \) in order to adjust their values to some resonance or suppression conditions, so as to favor the response associated with the relaxation time \( \tau_r \) over that associated with \( \tau_p \) originating from interfering reactants.

As far as instrumentation is concerned, the signal-to-noise ratio of the final signal \( \varphi \) varies as the square root of the number of periods \( n \).
3. Examples of Imaging Protocols Harnessing Reactive Modules

This section provides a comprehensive review of the publications on the various microscopies that exploit the kinetics of reactive modules to generate a contrast. The nature of the control parameter provides a guideline. Moreover, for both light and temperature, we first introduce the techniques dedicated to image reaction relaxation times. Indeed, the latter methods have often been used as starting points for further methodological developments.

3.1. Light as Perturbation Parameter

3.1.1. Fluorescence-Lifetime Imaging Microscopy (FLIM)

Quantitative in cellulo imaging of a fluorescent label requires careful calibrations, which must account for fluorophore concentration, bleaching rate, instrument settings (e.g. laser power, collection factors, detector gain). Although ratiometric measurements greatly simplify procedures, FLIM offers a more straightforward alternative.\textsuperscript{161,163–166} Lifetime is an intrinsic property of the fluorophore, it is independent of the local concentration and of the illumination parameters. Thus, its variations directly inform on the evolution of the molecular environment around the probe.\textsuperscript{161,162}

FLIM can be implemented in three different ways, all of them allowing a way to analyze the relaxation subsequent to light absorption for each pixel.\textsuperscript{219,220} First, time-domain acquisitions rely on a pulsed laser excitation, the photons being collected according to two possible modalities. In time-gated FLIM, fluorescence intensity is recorded after various delays following the excitation and the intensity decay is reconstructed from successive recordings.\textsuperscript{208} In time-correlated single-photon counting (TCSPC) FLIM, individual photons are detected and the associated delays between excitation and emission are measured, the intensity decay is then built up as the histogram of the arrival times.\textsuperscript{224} Next, fitting the temporal evolution of the fluorescence intensity with a single monoexponential or a more complex mathematical model gives access to information concerning the photophysical properties of the fluorophores. Although the instrument response function is typically around 100 ps, deconvolution algorithms enable one to measure lifetime with a resolution of a few tens of picoseconds.

On the other hand, frequency-domain FLIM exploits the use of modulated excitation light. It results in a fluorescence signal that is also periodically modulated, with a given modulation depth and a given phase delay relative to the excitation. The fluorescence lifetime is here determined from the values of the two latter parameters.\textsuperscript{218,231,232} However, multiple acquisition frequencies may be required to analyze complex decays.

More recently, a third group of techniques has been introduced, which lies between the time- and the frequency-domain methods. These stroboscopic microscopies, called strobe FLIM, rely on measuring the integrated emission intensity of a sample submitted to a train of excitation pulses (consequently bypassing the need for fast detectors). By performing several acquisitions at different repetition rates or pulse widths, it is then possible to extract lifetimes and transition rate constants by fitting.\textsuperscript{213–215}

In general, TCSPC FLIM is used on laser point scanning microscopes (confocal or multi-photon) while time-gated, frequency-domain and strobe FLIM are employed on wide-field microscopes equipped with cameras. Depending on the requirements and on the instruments, acquisition of FLIM images can take hundreds of milliseconds up to several minutes.

The unstable nature of the excited states make their electronic structure sensitive to the molecular context and the relaxation pathways towards the ground state can then be significantly affected by the environment. Hence, the use of FLIM in life sciences generally corresponds to the generation of images in which the contrast reports on the value of a local parameter,\textsuperscript{163,165–166} either a physical one (e.g. microviscosity\textsuperscript{236–238} or temperature\textsuperscript{238}) or a chemical one (e.g. concentration of oxygen,\textsuperscript{229,240} pH\textsuperscript{124} binding of biomolecules,\textsuperscript{208,213,242–244} conformational change).\textsuperscript{213–214}

3.1.2. Fluorescence-Lifetime Filtering

After having focused on the production of an image in which each pixel is associated with a collection of relaxation times, we now introduce the strategies that have been designed to enhance, or extinguish, the contribution of selected fluorophores in a mixture, according to their chemical nature or to their physicochemical environment. Gated acquisition enables one to emphasize the longer lifetimes since the fast-arriving photons are not collected (Figure 3a).\textsuperscript{213} Working in the time domain with such high-pass filters\textsuperscript{14} is for instance suitable to selectively detect labels like rare-earth ions (chelated\textsuperscript{205} or inside oxide nanoparticles),\textsuperscript{211} quantum dots,\textsuperscript{210} and nitrogen-vacancy centers in nanodiamonds.\textsuperscript{212} As far as the frequency domain and homodyne detection are concerned, behaviors get richer\textsuperscript{226,227} and the imaging protocols we discuss here owe much to previous modulation spectroscopy studies performed either to elucidate photochemical processes\textsuperscript{158,159,243} or to resolve spectra of compounds dispersed in mixtures.\textsuperscript{213,228,240} First, only the systems whose lifetimes are smaller than the inverse of the excitation frequency $\omega_p$ can display a significant oscillatory response. Thus, appropriately selecting the light modulation setting allows one to create a low-pass filter. Next, when demodulation is performed in delayed quadrature with the excitation (i.e. with $\psi = -\pi/2$), one obtains a band-pass filter which maximizes the contribution issued from labels satisfying $\tau_1 - 1/\omega_p$. Finally, it is also possible to suppress the signal arising from a given fluorophore by demodulating in quadrature with respect to its emission (i.e. with $\psi = \arctan(\tau_1 \omega_p) \pm \pi/2$),\textsuperscript{15} thereby realizing a band-stop filter.\textsuperscript{217,228,229,231,232,247}

\textsuperscript{14} By convention the filter nomenclature is here given with respect to the relaxation times that are either conserved or suppressed.

\textsuperscript{15} Lifetime suppression can alternatively be obtained by subtraction of images acquired with different phase angles.\textsuperscript{217,218,240}
Microscopies based on fluorescence-lifetime discrimination has enabled to selectively image small molecules bound to larger ones, for instance acridine orange intercalated in DNA with a time-domain setup\cite{208} (Figure 4a) or NADH complexed by the malate dehydrogenase with a frequency-domain one (Figure 4b).\cite{231}

### 3.1.3. Optical Lock-in Detection (OLID)

Several fluorescent labels can be reversibly switched between two states of different brightnesses by alternating illumination with two light sources of appropriate wavelengths. OLID has exploited this property for dynamic contrast generation in biological environments.\cite{221–223, 248, 249} More precisely, after several cycles of optical switching, the modulated fluorescence signal issued from the target is isolated from the non-modulated background by cross-correlation with a reference waveform. The latter may be provided by pixels corresponding to micrometer-sized beads coated with the optical switch—that have thus been added to the sample—or by the region of the imaged object that exhibits the highest depth of modulation.\cite{221} Both the fluorophores responsible for the endogenous background and the ones conventionally introduced for labeling (e.g. GFP) are not expected to exhibit optical switching; their emission is thus not correlated with the reference signal. Only the areas containing optical switches display a cross-correlation coefficient that approaches one; otherwise the value of the latter is close to zero.

The first demonstration of OLID relied on labels belonging to the families of the genetically encoded Dronpa proteins\cite{250, 251} and of the synthetic spiropyrans/merocyanins. Dynamic contrast imaging was achieved in living cells as well as in tissues of living Xenopus and zebrafish embryos (Figure 4c).\cite{221} OLID has subsequently been implemented with Spinach, a photoswitchable fluorescent RNA labeling tag which presents a fast thermally driven recovery after photoswitching and which consequently only requires the use a single modulated light source.\cite{252}

Other developments of OLID were focused on the use of fluorescence resonant energy transfer (FRET). The donor is a regular fluorophore whereas the acceptor is an optical switch.\cite{253} Several synthetic molecules\cite{254} and nanoparticles\cite{255} have been accordingly designed and characterized. This combined OLID-FRET approach was implemented to improve con-

![Figure 4](image-url)

**Figure 4.** Examples of kinetically filtered images obtained by relying on light as a perturbation parameter. The images on the left were obtained in classical fluorescence microscopy whereas the ones on the right were generated upon appropriate light excitation and kinetic analysis. a) Time-gated FLIM images of acridine orange stained polytene chromosome. Adapted from Figures 6a,b in Marriott et al.\cite{208} b) Lifetime phase-suppressed images and corresponding fluorescence intensity profiles of free and protein-bound NADH in cuvettes. \(F_1\) and \(F_2\) refer to free NADH at different concentrations, P–B to NADH bound to dehydrogenase, and POP to a standard fluorophore used as a reference. In contrast to the nonprocessed phase-sensitive image shown in the left, the right image shows that one can modulate the amplitude of the cuvette response to light modulation to specifically suppress the signals associated with lifetime \(\tau_p\). Adapted from Figure 10 in Szmacinski et al.\cite{247} c) OLID image of cis-Dronpa-actin within the growth cone of a motor neuron in a live, deskinned Xenopus embryo. Adapted from Figure 4c,d in Marriott et al. (Copyright (2008) National Academy of Sciences, U.S.A.).\cite{221} d) SAFIRE selective image of mitochondria-targeted AcGFP in the presence of nuclear targeted EGFP in NIH 3T3 mouse fibroblasts. Min to max color bar is 0 to 5.9 x 10$^5$ photon counts. Scale bar: 10 μm. Adapted from Figure 4a in Jablonski et al.\cite{253} e) OPIOM selective epifluorescence imaging of nuclear Dronpa-3 against membrane-localized EGFP in mammalian HEK293 cells. Min to max grey level scale is 0 to 4 x 10$^2$ and 0 to 5 x 10$^2$ in the left and right images respectively. Scale bar: 50 μm. Adapted from Figure 3a in Quérard et al.\cite{256}
trast in immunofluorescence imaging microscopy.\textsuperscript{[256]} It is also worth noting that such FRET systems enable one to switch between two colors, that of the donor and that of the acceptor, thereby allowing selective detection of the label by auto-correlation.\textsuperscript{[255]} They constitute an alternative to spiropyans/merocyanins dyes that have been synthesized to exhibit two-color modulation by switching a single molecular species.\textsuperscript{[244,257]}

Finally, the OLID strategy has recently been adapted to multispectral optoacoustic tomography (tuMSOT) in order to get high contrast imaging of reversibly switchable fluorescent proteins in the presence of highly absorbing blood.\textsuperscript{[258]}

3.1.4. Synchronously Amplified Fluorescence Image Recovery (SAFIRe)

In this protocol, the signal issued from the fluorophore of interest is modulated without modulating the background.\textsuperscript{[215]} A primary light source promotes the label from its ground state to its first singlet and triplet excited states whereas a second beam is used to depopulate the resulting transient long-lived dark states faster than they normally decay. Modulating the intensity of this secondary light source with appropriate frequency and amplitude thus permits the selective modulation of the populations of the excited states of the targeted molecule, ultimately modulating the emitted signal. The time trace of the photon flux arriving on the camera is typically analyzed by Fourier transform, yielding for each pixel the amplitude of the light oscillations as function of the frequency. The $\mathcal{Q}$ values obtained at $\omega_p$ has been shown to be proportional to the concentration of the targeted fluorophore, even in solutions exhibiting a high-level background.\textsuperscript{[216]} Indeed, the noisy as well as the constant components of the collected signal are filtered out during data treatment. Noteworthy, it has been possible to image labels up to 6 mm deep within a medium mimicking skin tissue, composed of highly scattering and fluorescent objects.\textsuperscript{[259]}

In SAFIRe, the choice of the wavelength of the secondary, modulated, excitation is crucial. Since endogenous fluorophores in biological media absorb mostly in the blue-green wavelength range, adopting longer excitation wavelengths limit the contamination of the useful signal with an oscillatory background. Furthermore, to avoid any additional fluorescence emission due to some direct excitation with the secondary light source, its wavelength is also red-shifted with respect to the collected fluorescence. As a consequence, the implementation of the present technique requires specific fluorophores. It has first been experimentally demonstrated with silver nanodots\textsuperscript{[214]} and organic fluorophores (xanthene\textsuperscript{[215]} and cyanine dyes).\textsuperscript{[260,261]}

In line with previously published data,\textsuperscript{[262]} two modulatable fluorescent proteins could be identified in order to implement SAFIRe in bioimaging. AcGFp targeted to mitochondria was used to selectively visualize these organelles against a background of non-modulatable EGFP, either present in the whole cytoplasm or localized at the nucleus (Figure 4d).\textsuperscript{[263]} Employing modBFP/H148K additionally enabled to reduce the auto-fluorescent background.\textsuperscript{[264]}

The need for specific labels can be seen as a limitation. Therefore, to further apply SAFIRe to fluorophores without any dark states, an approach in combination with FRET has been proposed.\textsuperscript{[260]} The acceptor is excited with the secondary, modulated, light source. As the laser intensity is important, the first excited state is nearly saturated, which yields an oscillating inhibition of the energy transfer. The donor is excited with the primary, constant, light source and it is the variations of its emission that are used for image computation. This concept was experimentally evidenced using a DNA hairpin whose extremities were labeled with either the 6-Fam/TAMRA pair or the Cy3/Cy5 one.

To similarly circumvent the requirement for dedicated fluorophores, developments relying on stimulated emission have also been proposed.\textsuperscript{[266]} The first excited state plays the role of the dark state and its population is controlled using a secondary laser whose wavelength is chosen to be longer than the fluorescence emission one, so as to prevent crosstalk.

3.1.5. Out-of-Phase Imaging after Optical Modulation (OPIOM)

This technique bears similarities with modulation excitation spectroscopy (see Section 2.1).\textsuperscript{[39,40]} It exploits the rich kinetic signature of a reversibly photoswitching fluorescent label to increase, both selectively and quantitatively, its contrast against a background of spectrally interfering species, photoswitchable or not.\textsuperscript{[25,220]} Filtering the specific contribution of the probe only requires a phase-sensitive detection set in delayed quadrature when the intensity and frequency of a modulated excitation light match the photoswitching dynamics of the targeted reactive module. After in vitro validation, OPIOM has been applied to specifically image several reversibly photoswitchable fluorescent proteins belonging to the Dronpa family in living mammalian cells and zebrafish embryos. Acquisition was achieved up to a 10 Hz video rate, with both epifluorescence and light sheet fluorescence microscopes (Figure 4e).\textsuperscript{[250,267]}

Noteworthy, the diversity of the photochemical properties of the currently available reversibly photoswitchable fluorescent proteins\textsuperscript{[216,251]} suggests that OPIOM could be useful for fast multiplexed observations under adverse conditions in biological samples. Indeed, appropriately tuning the frequency and the average intensity of the light source to resonant conditions permits to simply and selectively extract the contribution from a specific label even if other ones with similar spectral signature but distinct reactive module dynamics are present.\textsuperscript{[220,267]}

Finally, OPIOM benefits from improved spatial resolution since both the amplitude and the phase lag of the photoswitching response to light modulation depend on light intensity. Hence, the resonance conditions are only matched where the photon flux is optimum, which can be just at the center of the beam profile, yielding a narrower OPIOM response profile than the one of illumination.\textsuperscript{[25]}

3.2. Temperature as Perturbation Parameter

3.2.1. Chemical Relaxation Time Imaging

Similarly to what is done in FLIM, chemical relaxation time maps can either be generated in the time or in the frequency domain. They have been respectively obtained according to techniques which have been named FReI for fast relaxation imaging[56,67,74,268–270] and TOOL microscopy for temperature oscillation lock-in microscopy.[52,66] In both cases the reactive system is driven out of equilibrium by IR laser heating, the amplitude of the perturbation, either a jump or a sine wave, being of a few Kelvins. FReI can scrutinize a time window spanning from ~0.02 to 20 s whereas TOOL microscopy addresses the ~1 to 1000 Hz frequency range. The two methods are thus able to perform measurements over three decades and the relaxation times reported so far correspond to the centers of these intervals, around a few seconds for FReI and around a few tens of milliseconds for TOOL microscopy. Whatever the protocol, the extent of the investigated two-state exchange is determined using fluorescent labels whose brightness are altered upon transformation of one species into the other (FRET[56,66] or emitter-quencher pairs[52] are of common use). In addition, imaging is wide-field and kinetic data are extracted for each pixel of the camera mounted on an epifluorescence microscope. In FReI the relaxation time is straightforwardly determined by fitting the individual time traces with some appropriate exponential function. Conversely, TOOL microscopy relies on a more complex acquisition scheme where the fluorescent signal is analogically demodulated by varying the microscope light source intensity with the same periodicity as the heating laser one. More precisely, to measure both the amplitude and the phase of the chemical system response, one needs four images, obtained by setting the phase lag between the two oscillators at the four integral multiple values of π/2. The operation is repeated at various frequencies and for each pixel it yields a Bode diagram from which the relaxation time can be retrieved. Such a homodyne detection process is reminiscent of lock-in amplifiers[226,227] and an advantageous signal-to-noise ratio consequently results.[48,129]

As far as biophysics is concerned, both FReI and TOOL microscopy have been utilized to investigate the effect of molecular crowding on reactivity. In the first case the reporting reaction was the folding of a protein, either the phosphoglycerate kinase enzyme[56,67,74,268–270] or the VlsE surface lipoprotein,[103] fused at its extremities with AcGF1 and mCherry (Figure 5a). In the second case, use was made of the hybridization between two oligonucleotides labeled with rhodamine green and ROX (Figure 5b).[66] Parameters such as the reagent location (in the nucleus, in the cytoplasm, at the endoplasmic reticulum surface, or at the plasma membrane)[66,268,271] as well as the cell cycle stage[270] were found to influence significantly chemical kinetics at the molecular level.

3.2.2. Out-of-Phase Imaging after Thermal Modulation

Although temperature has been harnessed as a control parameter in several modulation spectroscopy works,[39,272–274] to the best of our knowledge, only some of the authors of the present review have proposed an application in quantitative imaging, devising a technique that presents much similarities with OPIOM.[34,90] Thermal harmonic forcing and frequency-domain acquisition were considered as a first step towards the selective detection of a compound in a mixture. Then we built a function of the observable that exhibits a resonant behavior for the reactive module associated with targeted dynamic properties. Although preliminary theoretical investigations were undertaken on enzymatic systems following Michaelis–Menten kinetics,[272] demonstration experiments were realized on two-state exchanging reagents obeying Equation (1).[34,90] It was shown that the reactive couple obeying k20 = k02 = ω2/2 displayed the maximum amplitude of concentration oscillations when the signal was acquired in delayed quadrature with the excitation. Practically, modulation of the temperature in a microfluidic chamber was driven by Joule dissipation in a thin film resistor,[83] concentration variations were recorded in the time domain for each pixel, and out-of-phase demodulation was implemented in silico. Small oligonucleotides bearing fluorophores (e.g. rhodamine green, Texas red) were utilized as model systems and adjusting the resonance conditions enabled us to titrate a given targeted sequence even in the presence of contaminating species (Figure 5c).[220] In a second set of experiments, the reaction was employed as a probe to reveal the existence of physical structures impacting on its kinetics.[90] Up to now no selective imaging in cells or tissues has been accomplished.

4. Concluding Remarks and Perspectives

There is presently an urgent need to introduce powerful non-invasive analytical tools fruitfully facing the complexity of biological samples. The preceding sections have shown that the use of discrimination criteria relying on reaction kinetics is highly promising for imaging living cells. Indeed, the theoretical framework is well established and a few protocols have been experimentally validated, thereby constituting as many starting points for future developments. In the following subsections, we emphasize the main properties provided by the explored strategies and draw a few avenues expected to lead to possible improvements.

4.1. Discrimination Selectivity

The degree of kinetics exploitation of the analytical process is one of the main factors governing the ability to image the targeted reactive module only. Some approaches, like OLID, are in fact much related to dynamic contrast imaging (see Section 2.1).[272] The final image directly reports on a label that is the sole to respond to a periodic excitation, which allows one to get rid from the noise as well as from the constant background issued from other inert species or from the illumination source. In contrast, most of the other techniques exploit the kinetic features of the targeted reactive module to tune their imaging conditions to further enhance discrimination. The targeted signal is now not only retrieved from noise and back-
ground but also from the contribution of other responsive modules.

More precisely, when one relies on analytical strategies making use of two-state exchange modules, selectivity results from discriminating relaxation times. Depending on the constraints applied to circumvent the targeted kinetic window, two different levels of discrimination can be obtained: 1) Low- and high-pass kinetic filters select the components exhibiting either the fastest or the slowest responses, respectively. For instance, in SAFIRE one images only the species reacting faster than the threshold defined by the frequency of illumination modulation.215,216 On the contrary, in time-gated FLIM one discerns all the fluorophores exhibiting lifetimes longer than the threshold fixed by the gate position.209–212 2) Band-pass filters select components possessing relaxation times located in a given kinetic window, which is typically one order of magnitude wide around 1/\( \tau_p \). Usually they make use of a phase-sensitive detection set in delayed quadrature with the periodic excitation, as in fluorescence-lifetime imaging \( \text{FLIM} \), in OPIOM225,226 and in out-of-phase imaging upon thermal modulation.24,90 Interestingly, for the two latter protocols a second “resonance” condition has to be fulfilled in addition to the preceding \( \tau_p \tau_f = 1 \) one. It thus enables to play with another setting, respectively the illumination intensity and the concentration of the titrating reagent, to be more restrictive on the species that will be detected.

An attractive avenue to improve discrimination selectivity could consist in the mobilization of reactive modules more complex than the two-state exchange. A first approach relying on \( n \) state exchanges, which give rise to \( n-1 \) relaxation times,223 could be used to target a given analyte by means of a multifrequency excitation of the control parameter. Some of us have already theoretically explored this strategy with \( n = 3 \) and showed that it is rather constraining in terms of the values of the rate constants if one wants the target to exhibit the most singular response.275 This limitation originates from the linearity of the kinetic equations ruling \( n \) state exchanges. In fact, more complex reactive schemes, possibly displaying nonlinearities, should provide more favorable opportunities to singularize a given reactive label.

Finally, it is advantageous to benefit from simple analytical “resonant” conditions for matching the settings of both the perturbative instrument and the acquisition system with the dynamics of the targeted module (see the case of OPIOM25,220 or the one of its thermal equivalent).24,90 However, selectivity then still depends on the reliability of the kinetic properties of the targeted reactive module since any change in reactive scheme topology or rate constants would be detrimental to optimal kinetic discrimination. Thus, the kinetic properties of the label should not significantly vary with the environment.

**4.2. Perturbation Parameters**

Numerous tools have been developed to control concentrations and investigate dynamic phenomena, or to influence their course, in a biologically relevant context. Yet, although...
modulating reactant quantities could constitute an interesting strategy to achieve kinetic discrimination, no account demonstrating its application to selective imaging has been reported so far. The only reported applications harnessing a similar principle were in vitro measurements performed in modulation spectroscopy.\textsuperscript{[29,40,225,276]} In cells, one may encounter hindered diffusion or non-specific interactions with cytoplasm components. When using a photoactivatable precursor, precise evaluation of the excitation amplitude would be an issue since calibration of photorelease efficiency is difficult (see Section 2.2.1). Finally, it would be often a challenge to sequentially control, in a same experiment, both increases and decreases of concentrations.

Similarly, even if significant technical advances have recently been made,\textsuperscript{[52, 56, 66, 74, 268]} temperature modulations have been only marginally used in microscopies exploiting reaction kinetics.\textsuperscript{[24, 50]} Heat diffuses freely through cell structures and thus the limitations that impede molecular transport should not apply. However, most titrations exploit bimolecular interactions, which implies that the labeled reporting reagent would still have to reach the molecule that one wants to quantify.

Thus, apart from experiments related to easily accessible membrane receptors, we anticipate that in the near future both concentration and temperature will mainly be used as control parameters for in vitro kinetically filtered detection, with surface sensors for instance.\textsuperscript{[112, 113]}

As a conclusion, light will probably remain the most favorable control parameter to design new imaging protocols exploiting reaction kinetics. Beyond the advantages for cell microscopy it provides (and which have been already underlined above), light should indeed benefit from the continuous development of photoactivable materials (including from biological origin). These innovations will permit to envision complex photophysical or photochemical behaviors prone to the conception of more discriminative schemes. Furthermore, the implementation on commercially available microscopes of some of the presently discussed techniques (e.g. OLID, OPIOM) does not require any extensive, expensive, nor complicated modification. This feature should thus also motivate the elaboration of parent imaging modalities.

4.3. Observables

The constraints are severe to image low-abundance cellular components with kinetic filters. Therefore, one can hardly expect other spectroscopies than the ones reported in this review to be used to that purpose. In contrast, in order to improve the discrimination efficiency, one can imagine to simultaneously collect either more observables or richer ones. As an example, we recently showed that recording the second-order concentration response of reagents submitted to a temperature modulation could be more informative than simply performing first-order data acquisition.\textsuperscript{[69, 71]} Finally, of particular interest would be to introduce optical probing techniques acting as chemical amperometers to non-invasively evaluate reaction fluxes in situ in living cells.\textsuperscript{16}

4.4. Contrast Generation Modalities

The protocols dedicated to the production of images emphasizing the kinetics of reactive modules are rather similar to the ones used in dynamic contrast imaging.\textsuperscript{[17, 227]} Nevertheless, several differences exist among the various techniques we have introduced.

First one needs to consider their requirements for a reference signal. Usually, only synchronization of the observable \( O(t) \) with the control parameter \( P(t) \) is needed: for instance, a trigger fixes time zero in the time domain\textsuperscript{[208–210, 219]} or some device locks the phase in the frequency domain.\textsuperscript{[52, 212, 218, 240]} However, protocols relying on cross-correlation, such as OLID\textsuperscript{[221]} are more demanding since they necessitate to acquire the whole temporal response of the pure label in order to use it as a reference signal to selectively retrieve the label contribution in the other pixels of the image. In fact, only auto-correlation appears to yield reference-free analyses.\textsuperscript{[224]}

Secondly, the methods are also dissimilar in their ability to reflect the concentration of the targeted reactive module. The filtered signal \( \tilde{O} \) is proportional to the actual amount of the label with imaging protocols such as SAFIRe\textsuperscript{[216]}, OPIOM\textsuperscript{[220]} or the thermal equivalent of the latter.\textsuperscript{[24]} In contrast, such a proportionality is lost for some other techniques like OLID\textsuperscript{[221]} even if quantitative information could in principle be extracted from the raw data but it has not been demonstrated yet.

Finally, fast image acquisition is crucial when observing living cells. In classical optical microscopies, which are based on the detection of a signal intensity, the temporal resolution \( \tau_\text{τ} \) is imposed by the required signal-to-noise ratio (a few microseconds per pixel is typically necessary in fluorescence confocal microscopy). In contrast, when exploiting kinetic for video acquisition, imaging speed becomes limited by the relaxation time of the targeted reactive module, \( \tau_\text{r} \), and by the protocol utilized to generate contrast. Hence, in techniques relying on a photophysical exchange between the ground state and the first excited one \( \tau_\text{r} \), lies in the nanosecond range, which gives access to submillisecond dynamics when the label concentration is large enough. Slower acquisition rate are obtained for microscopes making use of long lifetime non-emissive states (SAFIRe, gated detection) or photoisomerizations (OLID, OPIOM). Moreover, any imaging protocol relying on bimolecular reactions should be limited to a time scale of a few hundreds of milliseconds at best since reagents are not expected to be utilized at concentrations higher than a few tens of micromolar in a living cell.\textsuperscript{[24, 66]}

\textsuperscript{15} Isotopic dilution methods relying on radioactivity counting or NMR are presently used to get such an information.\textsuperscript{[217, 276]}

\textsuperscript{16} In the latter group, differential analysis must nevertheless be considered apart. Indeed it only relies on removing the background recorded without stimulation from the data collected under a constant \( P \) value and it does not enable to discriminate against the phenomena associated with various relaxation times \( \tau \).

Keywords: cell imaging · chemical kinetics · dynamic contrast · imaging protocols · reactive modules
