Multiple fluorescent probes (multi-dyes) and multi-laser configurations can significantly extend the applications and accuracy of flow cytometry (Refs 1-5), as well as other applications in biology including imaging (Ref 6) and microscopy (Ref 7). Multiple fluorescent probes enable the user to identify more than one target, but difficulties can arise due to overlapping spectral emissions of the different probes. In particular, spectral overlapping of fluorescent and phosphorescent emission signals can lead to critical analysis errors, which can potentially result in a misdiagnosis Existing compensation techniques (Refs 4,5) may not give accurate results for overlapping spillovers of more than 50% (typical scenario for probes with strong phosphorescence). An accurate modeling tool should be used to predict such errors during both designing and screening stages.

Existing commercial modeling tools may not directly model recently introduced dyes that undergo multi-photon excitation (Ref 8), requiring some modifications to the math model and the code.

This application note shows how our modeling software SimphoSOFT® can effectively and quickly enable the user to optimize a selection of probes or, at a minimum, helps to decide which probes should not be used at all in some experiments. Originally designed for modeling laser beam interactions with photo-activated materials in general, SimphoSOFT is particularly effective in solving problems encountered in the area of multiple fluorescence probes. Starting with version 3.0, SimphoSOFT can calculate the intensity of emission spectra of photo-activated dyes, which allows users to

- accurately estimate fluorescent and phosphorescent signals, which are calculated separately, and prevent mistakes from using potentially flawed or incomplete compensations methods in cases of multi-dye and multi-laser configurations.
- develop new and optimize existing dye sets for flow cytometry making the procedure less harmful and more accurate.
- be ahead of competitors by analyzing and including trendy multi-photon dyes to the existing dye sets when they are biologically compatible.

SimphoSOFT – Flow Cytometry Simulations Including Emission of Both Fluorescence and Phosphorescence

In flow cytometry, cells labeled with fluorescent probe molecules (dyes) are transported single-file in a liquid stream passing through a laser light beam. The fluorescence and phosphorescence that are emitted by each cell are filtered and sampled by an array of detectors. In many cases, a single light source and one type of probe molecule are used. However, additional information can be obtained from a sample if several different laser wavelengths and multiple probes fluorescing at multiple wavelengths are used. In some cases, overlapping portions (spillover) of the various fluorescent and phosphorescent spectra can occur in a single detector channel and can lead to analysis errors.

Currently, time-consuming methods are used to eliminate overlap as much as possible. A common technique is compensation in which the fluorescence from all but the target probe is measured in every detector. This procedure is done repeatedly until the contributions from every probe are measured in every detector. An $N \times N$ matrix is formed for N probes where the off-diagonal elements are the measured overflow. A linear algebra method is used to correct the measured signal by inverting this matrix and applying the N appropriate linear equations. This method is cumbersome and, in some cases incorrect, i.e. when the desired signal is small compared to the fluorescence of the

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unwanted probe. However, if a computer modeling tool could calculate the number of excited electrons, and, correspondingly, their emission, then it may be possible to obtain a good understanding of potential spectral overlap and reduce the compensation. Ideally, such a simulation would enable the user to select the optimal set of fluorescent probes for the various lasers used in the experiment. The ability of a computer tool to rapidly assess a host of dye-laser combinations could save considerable time and money.

SimphoSOFT uses a numerical method that descretizes both time and space into small intervals so that it can calculate on a near-instantaneous basis the absorption of laser light, electron populations and emitted light. It can then calculate the intensity of the emitted signal and determine the overlap of the spectra. This is something that cannot be done using linear algebra.

Using our unique numerical technique, SimphoSOFT can calculate the multispectral emissions (fluorescence and phosphorescence) for a cell containing one or many dyes. In the examples shown below, simulations will be done for cells containing either the commonly used dye molecule Alexa 488 or the dye molecule Alexa 647. Alexa 488 has high fluorescence quantum yield (QY = 0.92) and correspondingly low phosphorescence emission. In contrast to Alexa 488, Alexa 647 has low fluorescence quantum yield (QY = 0.33) and high phosphorescence quantum yield, resulting in potentially large phosphorescent emission at longer wavelengths. Note that in the examples, we will assume only two pathways for excited electrons, either fluorescence directly to the ground state or intersystem crossing to the triplet state followed by phosphorescence. For Alexa 647, the resulting phosphorescence spillover may be recorded in detector channels set aside for fluorescence emission from other dyes and can result in analysis errors.

For analyzing multi-dye configurations, the emission from Alexa 532 has been also calculated in the examples.

SimphoSOFT is an ideal simulation tool to accurately predict fluorescence and phosphorescence processes that can occur when multiple probe molecules are used. It will allow the user to better understand potential analysis problems and better optimize fluorescence probe selection to reduce emission overlaps.

Flow cytometry experiment

A typical commercial flow cytometer consists of several parts as shown schematically in Figure 1. Cells with attached fluorescent probes flow, usually single-file, through a narrow chamber. The dyes are excited by one or more lasers focused on the cell. In addition to scattered light, each dye emits fluorescence and phosphorescence, which are separated by dichroic splitters and band-pass filters, and finally detected by photomultiplier tubes or diode detectors.

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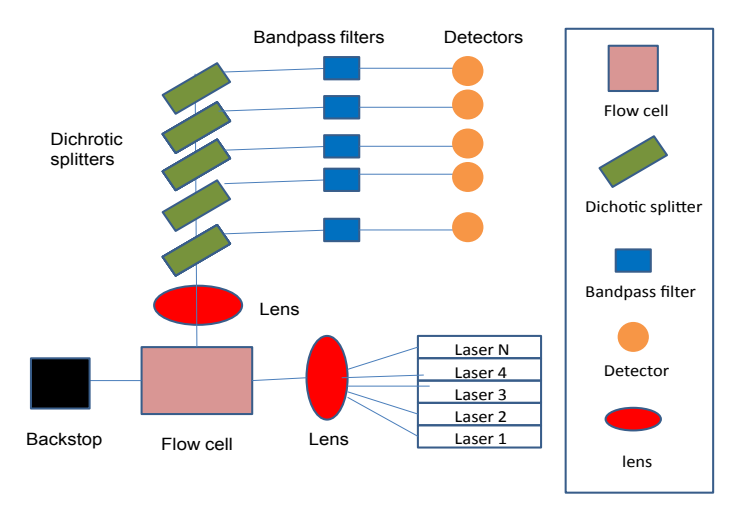
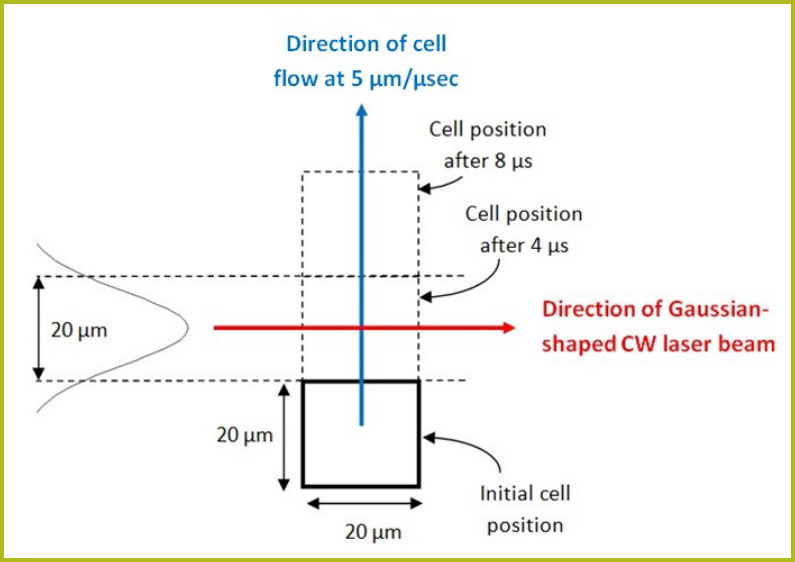
Figure 1. Schematic diagram of a typical flow cytometer



Flow cytometer simulations using SimphoSOFT

While different flow cytometers use various flow rates, lenses, filters and geometric optics, in the example simulations shown below, we assume the simple case of a single biological cell with dimensions 20 μm × 20 μm × 20 μm that flows though a stationary, Gaussian-shaped, continuous (CW) laser beam as in Figure 2(a). Some of the simulation parameters are listed in Figure 2(b). In this example, the cylindrical stationary laser beam has a radius of 10 μm (diameter of 20 μm) and a CW power of 15 mW.

Figure 2. A simplified flow cytometry model



(a)

Parameter	Value
Flow rate	5 μm/μs
Laser beam radius	10 μm
Sample path length	20 μm
Dye concentration	1×10^{16} cm^{-3}
Laser power	15 mW
Laser temporal duration	CW
Light duration on each portion of the cell	~4 μs

(b)

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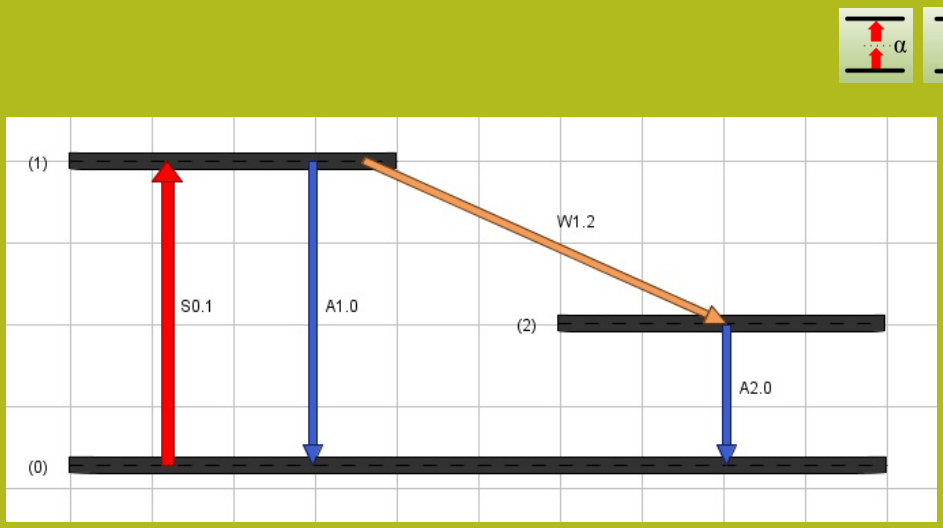
The cell moves at 5 μm/μs through the Gaussian-shaped laser beam. Each part of the cell sees the main part of the laser beam for approximately 4 μs. Note that a moving cell passing through a CW beam with Gaussian-shaped radial dependence in 4 μs is equivalent to having a stationary cell exposed to a 4 μs laser pulse that has Gaussian-shaped time-dependence. We will simulate the latter equivalent case.

Example of SimphoSOFT calculations

We will simulate a single-cell sample containing one type of molecule dispersed in a host material: Alexa 488, Alexa 532 or Alexa 647 molecules. The molecules have two important singlet energy states, labeled 0 and 1, and one triplet state, labeled 2, for optical transitions. A triplet state is not included explicitly in the conventional 2-level models. In SimphoSOFT, adding a new level and corresponding relaxation transitions is a very easy and fast process, which does not require updating the corresponding rate equations manually – SimphoSOFT will update them automatically. An APPENDIX at the end of this document contains a simple illustration on how one can add more levels and transitions to the existing energy level diagram in SimphoSOFT Material CAD (M-CAD).

The energy level diagram applied for Alexa 488, Alexa 532 and Alexa 647 is shown in Figure 3. Absorption transition S0.1 is a single-photon absorption from the singlet ground state 0 to a singlet excited state 1. Non-radiative relaxation (W1.2) is intersystem crossing (ISC) from the singlet excited state 1 to a triplet state 2. The possible radiative relaxations are fluorescence (A1.0) from state 1 to state 0 and phosphorescence (A2.0) from triplet state 2 to the ground state 0.

Figure 3. Screenshot of SimphoSOFT® M-CAD with energy level diagram of Alexa molecules 488, 532 and 647 with the graphical icons needed to create the diagram



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Description of the photophysical and laser parameters.

Table 1. Photophysical parameters of Alexa 488, Alexa 532 and Alexa 647

Material	Quantum Yield	λ (nm) (S0.1)	σ (cm^2) (S0.1)	τ_F (ns) (A1.0)	τ_{ISC} (ns) (W1.2)	τ_P (µs) (A2.0)
Alexa 488	0.92	488	2.549×10^{-16}	4.1	46	6.66[1]
Alexa 532	0.61	532	2.939×10^{-16}	2.5	3.9	6.66[2]
Alexa 647	0.33	640	6.85×10^{-16}	1.0	0.49	6.66[2]

[1]Measured, [2]Estimated

In cells containing Alexa 488, fluorescence emission will dominate over phosphorescence (see Figure 7.a). Phosphorescence is more significant in cells containing Alexa 532 (see Figure 7.b). This phosphorescence could be used as a distinguishing factor when cells potentially having different dyes need to be separated (as would be in the case for distinguishing Alexa 488 and Alexa 532). In cells containing Alexa 647, phosphorescence emission can be larger than fluorescence (see Figure 7.c). If another dye is used with Alexa 647 in a flow cytometry experiment, it is possible for the phosphorescence from Alexa 647 to be detected as a large signal in an adjacent fluorescence channel for the other dye. This is exactly the case when a filter from 750 to 800 nm is used to detect signals from Alexa 647 and Alexa 750 (not described here; for more details see our publication, Ref. 9).

We used the following laser beam parameters for simulations of beam propagation through samples of Alexa 488, Alexa 532 and Alexa 647. As was mentioned before, the incident beam has a Gaussian shape in both time and radius.

Table 2. Laser properties used in SimphoSOFT Alexa 488, Alexa 532 and Alexa 647 simulations

Laser parameter	Value
Pulse energy	0.06 µJ
Pulse radius (HW1/e^2M)	10 µm
Pulse FWHM	4 µs
Laser wavelength	488 nm (for Alexa 488) 532 nm (for Alexa 532) 640 nm (for Alexa 647)

Results of SimphoSOFT simulations of energy level populations of Alexa 488 during the light pulse

First, we run propagation simulation for an Alexa 488 sample. As the quantum yield is very high for this molecule, we expect that fluorescence will account for the most of the emission.

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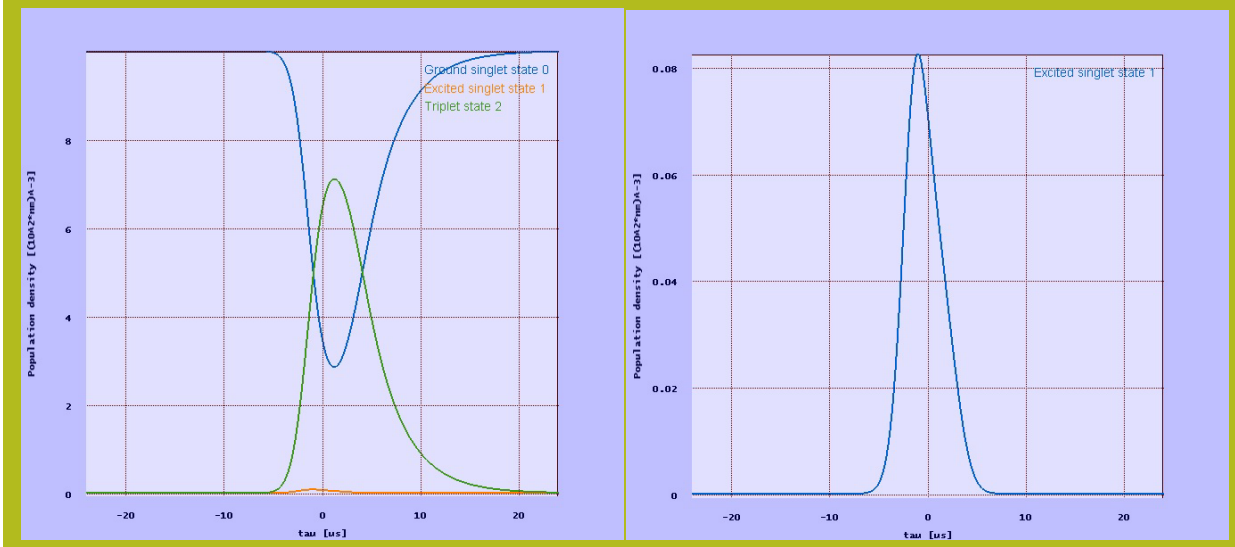
Figure 4. Results of SimphoSOFT simulation of Alexa 488 electronic population densities



(a) (b)

Figure 4(a): Graph of the populations of the three energy levels of Alexa 488 calculated at the radial center of the laser pulse. The temporal center of the pulse is at time tau = 0 μs in the figure. The ground singlet state 0 is partially depleted by the laser beam (blue curve). Electrons are pumped to excited singlet state 1 that has a low transient population due to the fast fluorescence rate ($1/\tau_F$) and is barely visible at the bottom of this graph (orange curve). Note that much of the phosphorescent light emitted by the long-lived triplet state 2 (green curve) will occur several microseconds after the cell passes the flow cytometer detector optics and will not be recorded by the flow cytometer detectors.

The magnitudes of fluorescence and phosphorescence signals are proportional to both the corresponding population densities of the excited singlet state 1 and the triplet state 2, respectively, and to the corresponding relaxation rates. The resulting fluorescence signal of Alexa 488 is greater than the phosphorescence signal (see Figure 7.a) due to the 3 orders of magnitude higher fluorescence rate ($1/\tau_F$) compared to the phosphorescence rate ($1/\tau_P$) (see the values of τ_F and τ_P in Table 1).

Figure 4(b): Graph of the population of excited state 1 for Alexa 488, expanded approximately by a factor of 10^2 compared to Figure 4(a). Once excited, an electron in state 1 will relax to the triplet state 2 with a low probability. Most of the electrons in state 1 will return to the ground state in a few nanoseconds and can then be re-excited to state 1 several times during the 4 μs time in the laser beam. The fluorescence quantum yield is very high for Alexa 488.

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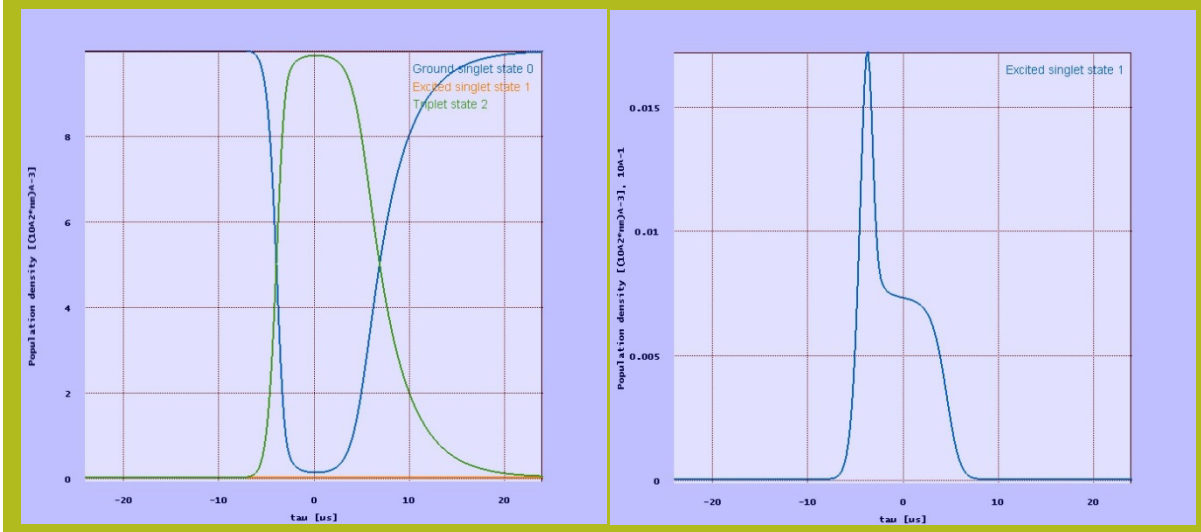
Results of SimphoSOFT simulations of energy level populations of Alexa 647 during the light pulse

In contrast to Alexa 488, the fluorescence quantum yield of Alexa 647 is low. We expect that this will result in phosphorescence dominating the fluorescence.

Figure 5. Results of SimphoSOFT simulation of Alexa 647 electronic population densities



(a) (b)

Figure 5(a): Graph of the populations of the three energy levels of Alexa 647 calculated at the radial center of the laser pulse. The temporal center of the pulse is at time, tau = 0. The ground singlet state 0 is quickly depleted (blue curve). Electrons are preferentially pumped via excited state 1 (orange curve) to the long-lived triplet state 2 (green curve). The excited singlet state 1 has a low transient population that is too small to be viewed on the scale of this graph.

Note that much of the phosphorescence emitted by electrons in the long-lived triplet state 2 will occur several microseconds after the cell passes the flow cytometer detector optics and will not be recorded by the flow cytometer detectors. Also, the ground state is almost depleted around half-way of the pulse. Such saturation would prevent a linear increase in the emission versus input energy after a certain critical value.

Figure 5(b): Graph of the population of excited state 1, expanded approximately by a factor of 5×10^3 compared to Figure 5(a).

The population of excited state 1 initially grows, but then drops and plateaus as the ground state is depleted. During the plateau, electrons return to ground state 0 by the 1.0 ns relaxation pathway and get re-excited several times to excited state 1. Once excited, an electron that is in excited state 1 will transfer to state 2 twice as often as going back to state 0 and will get trapped in state 2 for longer times, i.e. several microseconds (compare the corresponding fluorescence and inter-system rates τ_F and τ_{ISC} in Table 1). This will lead to the dominance of the phosphorescence signal (see Figure 7.c).

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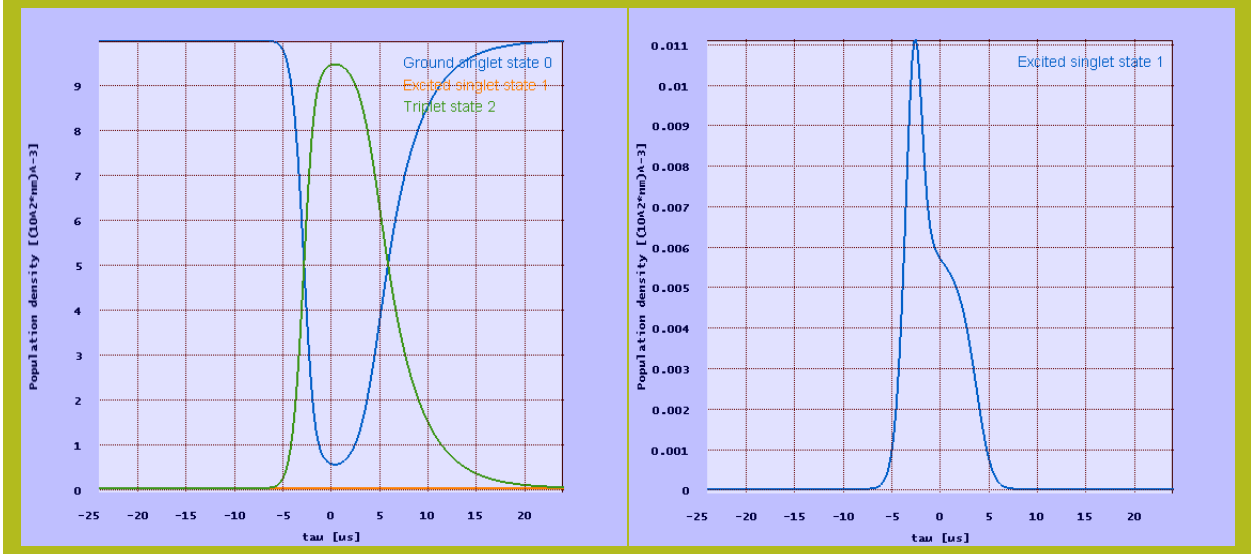
Results of SimphoSOFT simulations of energy level populations of Alexa 532 during the light pulse

Alexa 532 reveals a similar photo-physical behavior as Alexa 647. One important difference is that the fluorescence is still a dominant process. However, the dominance is not as profound as in the case of Alexa 488.

Figure 6. Results of SimphoSOFT simulation of Alexa 532 electronic population densities



(a) (b)

Figure 6(a): Graph of the populations of the three energy levels of Alexa 532 calculated at the radial center of the laser pulse. The temporal center of the pulse is at time, tau = 0.

The ground singlet state 0 is partially depleted (blue curve) but does not saturate completely. Some electrons are pumped via excited state 1 (orange curve) to the long-lived triplet state 2 (green curve). The excited singlet state 1 has a low transient population that is too small to be viewed on the scale of this graph.

Figure 6(b): Graph of the population of excited state 1, expanded approximately by a factor of 1×10^3 compared to Figure 6(a).

The population of excited state 1 initially grows, but then drops and somewhat plateaus. During the plateau, electrons return to ground state 0 by the 2.5 ns relaxation pathway and get re-excited to excited state 1. Once excited, an electron that is in excited state 1 will transfer to state 2 with around 40% probability and will get trapped in state 2 for longer times, i.e. several microseconds. This will lead to a substantial increase in the phosphorescence signal (see Figure 7.b) if compared with Alexa 488, but will not be that substantial as in the case of Alexa 647.

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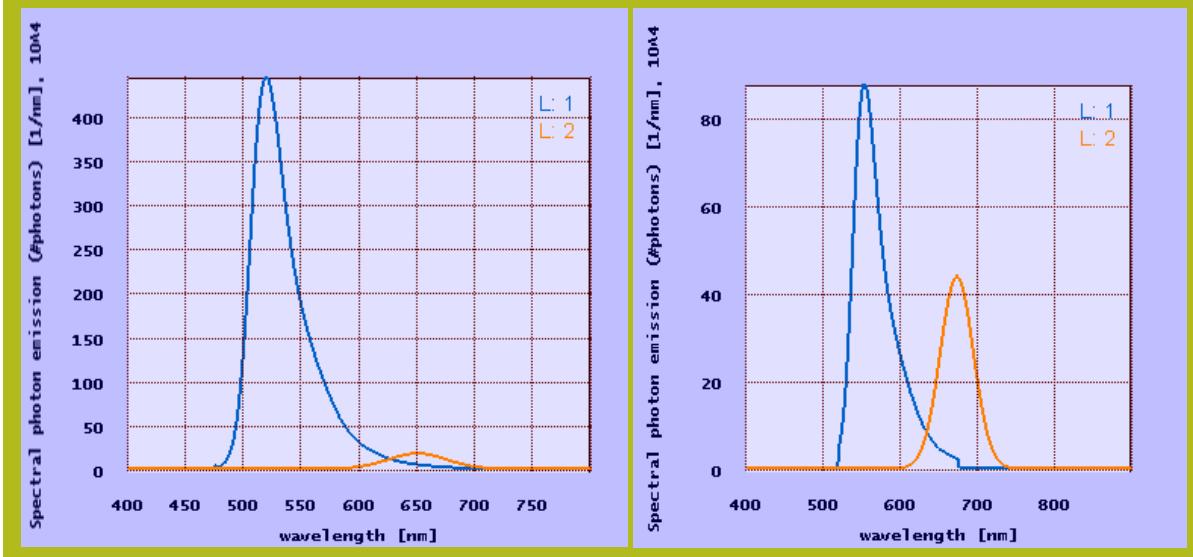
Results of SimphoSOFT simulations of light emission (fluorescence and phosphorescence) from Alexa 488, Alexa 532 and Alexa 647

SimphoSOFT can calculate the emission spectra of radiative transitions by numerically estimating the number of photons emitted per nanometer as well as by calculating the total number of photons emitted by each radiative transition (e.g., fluorescence transition from the first excited singlet state, or phosphorescence transitions from the lowest-energy triplet state). The shapes of fluorescent emission spectra of many types of molecules can be downloaded from 'Fluorescence-SpectraViewer' that is on the Invitrogen website at the link: http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Analysis/Labeling-Chemistry/Fluorescence-SpectraViewer.html. We obtained the flurorescence emission shape information for Alexa 488, Alexa 532 and Alexa 647 into SimphoSOFT to model[1] the radiative emission transition A1.0 (see Figure 3).

Phosphorescence emission spectra are not readily available. We approximated phosphorescence spectra in SimphoSOFT with a Gaussian function having a 50 nm width and with peaks red-shifted approximately 100 nm from the corresponding fluorescence peaks. This is consistent with similar organic molecules.

The resulting calculated spectra for Alexa 488, Alexa 532 and Alexa 647 are shown in Figures 7 (a), (b) and (c), respectively. The spectra represent the total emission in all directions. Only a portion of the emitted light will be collected by the flow cytometer detectors in an actual experiment. Depending on the parameters of the band-pass filters, the detectors can be configured to measure emission signal within a target wavelength range.

Figure 7. Results of SimphoSOFT simulation of emission spectra of Alexa 488, Alexa 532 and Alexa 647



(a) (b)

[1] SimphoSOFT Manual Help has a special topic 'Emission spectra for radiative transitions' (located in 'Concepts and Dialogs' \ M-CAD \ 'Working with M-CAD Workspace' section) which describes in detail how to setup emission spectra calculations.

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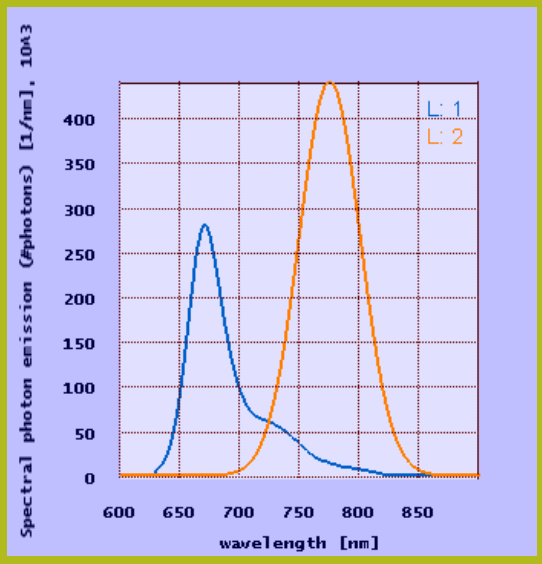

(c)

Figure 7(a): Alexa 488 fluorescence emission (blue curve) and phosphorescence emission (orange curve) from the biological cell as calculated by SimphoSOFT. The total integrated photons emitted in the fluorescence peak are calculated by SimphoSOFT to be 2.2×10^8 and the total integrated photons emitted in the phosphorescence peak are calculated to be 1.1×10^7. In this example, fluorescence emission is much larger than phosphorescence emission. Any phosphorescence that is emitted in the 600-700 nm spectral range is relatively small and can probably be handled by normal compensation methods.

Figure 7(b): Alexa 532 fluorescence emission (blue curve) and phosphorescence emission (orange curve) from the biological cell as calculated by SimphoSOFT. The total integrated photons emitted in the fluorescence peak are calculated by SimphoSOFT to be 4.7×10^7 and the total integrated photons emitted in the phosphorescence peak are calculated to be 2.3×10^7. In contrast to Figure 7(a), this figure shows that the phosphorescence emission is comparable with the fluorescence emission. The phosphorescence emission in the 625-725 nm spectral range may be large enough to cause problems during data analysis and normal compensate methods may not be accurate for detector channels located in that spectral region.

Figure 7(c): Alexa 647 fluorescence emission (blue curve) and phosphorescence emission (orange curve) from the biological cell as calculated by SimphoSOFT. The total integrated photons emitted in the fluorescence peak are calculated by SimphoSOFT to be 1.4×10^7 and the total integrated photons emitted in the phosphorescence peak are calculated to be 2.8×10^7. In contrast to Figure 7(a), this Figure shows that the phosphorescence emission is larger than fluorescence emission. The phosphorescence emission in the 725-825 nm spectral range may be large enough to cause problems during data analysis and normal compensate methods may not be accurate for detector channels located in that spectral region.

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Table 3. Emission signal from Alexa 488, Alexa 532 and Alexa 647

Material	Fluorescence (#photons)	Phosphorescence (#photons)	Total emission (#photons)
Alexa 488	2.2×10^8	1.1×10^7	2.3×10^8
Alexa 532	4.7×10^7	2.3×10^7	7.0×10^7
Alexa 647	1.4×10^7	2.8×10^7	4.2×10^7

A simple analysis below shows how important it is to choose correct sets of probes and filters to accurately interpret a signal detected in multi-dye flow cytometry. Spectral emission signals from all three dyes have been superimposed in one graph[2] shown in Figure 8.

Figure 8. Spectral photon emission of Alexa 488, 532, 647

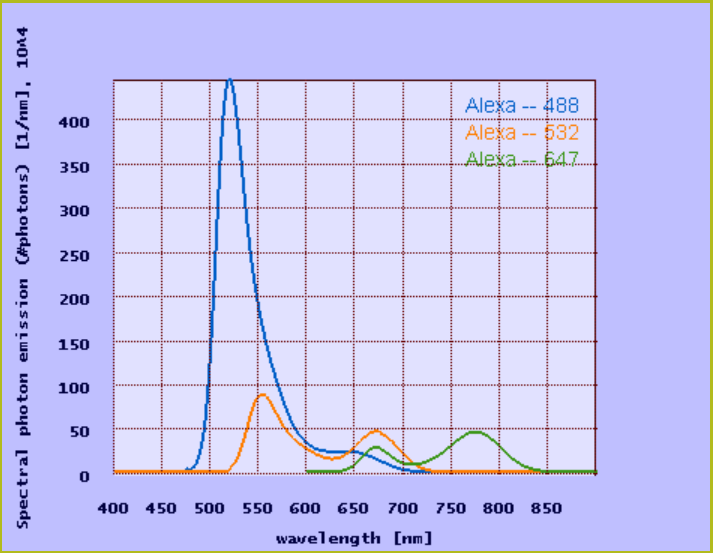

Figure 8: Spectral photon emission of Alexa 488 (blue curve), Alexa 532 (orange curve) and Alexa 647 (green curve), resulted in excitation by 488 nm, 532 nm and 640 nm lasers, respectively, of the same incident pulse energy (see Table 2).

Different combinations of these dyes require different strategies for accurate detection of the individual dyes. In a combination Alexa 488/647, the best way to separate the signals could be to use 475-575 nm band-pass filter to detect Alexa 488 and to use 750-850 nm band-pass filter to detect Alexa 647. For the former, the fluorescence part of the emission signal will be used, while for the latter, the phosphorescence signals will be used. The same strategy can be used for the combination Alexa 532/647. However, finding the right set of three filters for an Alexa

[2] Starting version 3.1, SimphoSOFT allows accumulating emission curves calculated from all the excited energy levels into one curve (for the case of Alexas molecules, it will accumulate fluorescence and phosphorescence into one curve). To be able to do that one should select 'Accumulate all curves' menu item of the corresponding EMS node in the 'Data Workspace' panel of SimphoSOFT Plot Creator dialog.

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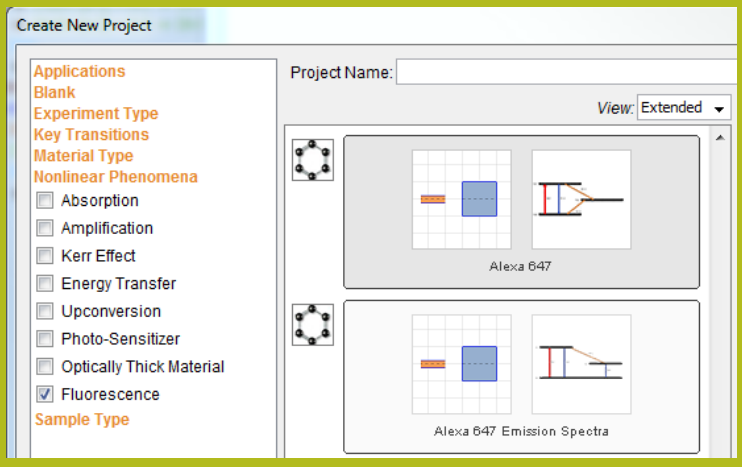
488/532/647 combination will be a quite challenging task, if resolvable at all. For example, a 625-725 nm band-pass filter may be a bad choice as it may pick up signals emitted from all three molecules.

SimphoSOFT[3] provides template projects to run these types of calculations. After the program is launched, one should select 'File \ Create New Project' menu item to open the SimphoSOFT template dialog. In the dialog, 'Fluorescence' category should be chosen within 'Nonlinear Phenomena' category group by clicking the group name and checking off the fluorescence category. The dialog will show all the templates available to model emission spectra in the center panel. After selecting a desired project from the list and entering the project name, one can proceed with creating a sample project and running emission calculations.

Figure 9: SimphoSOFT template dialog containing sample projects which have been used to prepare examples for this note



When the samples runs are completed, one can go back to E-CAD or M-CAD and change all necessary parameters to change the experimental conditions. Within M-CAD one can add more energy levels and electronic transitions to model more complicated cases (e.g., for the cases when intensity is high). SimphoSOFT adjusts the numerical engine appropriately to such changes.

SimphoSOFT is your solution!

The results of these runs show that the research staff should be careful in choosing the right combination of the dyes and lasers for flow cytometry. A significant overlap of phosphorescence spectra with fluorescence spectra can make existing compensation techniques inaccurate, which will lead to potential false positives during an actual flow cytometry session. Inaccuracies during flow cytometry can lead to undesirable wrong conclusions about a patient's condition.

SimphoSOFT Emission spectra feature[4] allows users to accurately estimate the emission spectra from different dyes in a chosen fluorophore set (dye set). It is extremely important in optimizing the existing sets or in designing new

[3] SimphoSOFT Alexa dye templates are available starting version 3.1
[4] SimphoSOFT emission spectra is available starting version 3.0

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sets. The user can alternate incident laser intensity (irradiation duration and beam spot) and wavelength during numerical simulations to estimate fluctuations. It is helpful in designing new dye sets and pre-screening the existing dye sets for different laser conditions. SimphoSOFT enables a multi-beam mode, which is essential in optimizing large fluorescent-dye sets. Without SimphoSOFT, a researcher would need to manually pre-test a potentially large number of dye sets to be able to come up with an optimal combination. SimphoSOFT will help you to reduce reagent and antibody costs, reduce time-consuming compensation measurements, and enable greater confidence in your measurements.

Multi-photon flow cytometry is becoming more and more popular bringing many advantages over single-photon excitation (Ref 8). SimphoSOFT is currently the only commercially available tool which does not require modifications to its math model to work with a wide class of multi-photon absorbers and laser combinations. Transition S0.1, shown in SimphoSOFT M-CAD snapshot of Figure 3, can be easily replaced by two-photon or three-photon absorption transition. Provided that the corresponding absorption cross-sections are known, emission signals from multi-photon absorbers can be analyzed in the same way as was done here for single-photon absorbers to determine the best solution. SimphoSOFT will help you to be ahead of everybody by modeling these new types of absorbers, more efficiently and with less experimental hazards, and bringing them to the market.

APPENDIX – Extending a conventional two-level fluorescence model with SimphoSOFT.

Extending a two-level fluorescence model of fluorescent probes to include a triplet state is very important for an accurate analysis of spectral emission. We show how easy it is to include extra energy levels and transitions to the energy level diagram in SimphoSOFT.

We start from the standard two-level system shown above. Material CAD (M-CAD) contains the energy level diagram in SimphoSOFT. Above is the ground state (0) of the probe dye molecule and above is the first excited (singlet) state (1) of the molecule. The molecule gets excited to this state by absorbing one photon of certain wavelength, which is represented by a red absorption transition S0.1 going from (0) to (1). Blue relaxation transition A1.0 represents a relaxation event which relaxes an excited molecule to its ground state (0).

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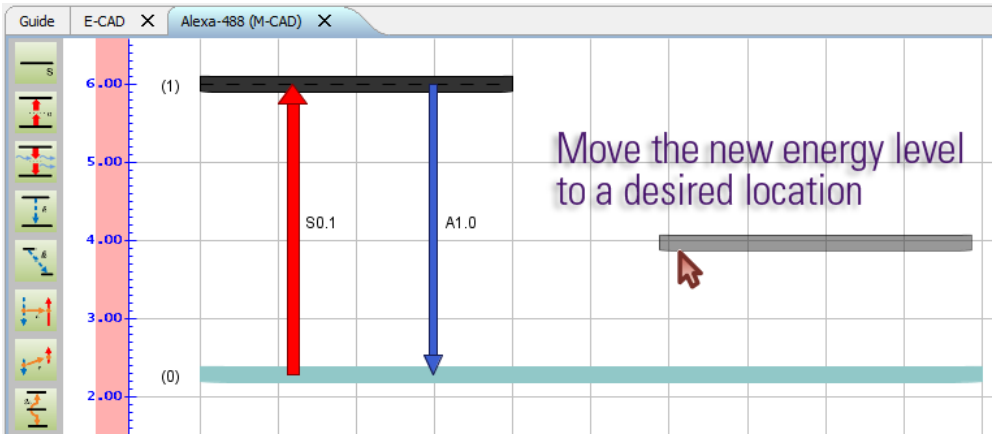
To include a new triplet state to the original energy level diagram (with the goal of taking into account phosphorescence) one should click the energy level icon located on the top of the icons bar (see above). The newly created energy level can be moved anywhere in M-CAD workspace (see below). The next click will finalize the level placement. One should then go to the energy level 'Properties' (right click with the pointer positioned on state (2)) and set the 'Energy' of state (2) to a value between the values for state (0) and state (1).



Now, one can add a new transition from the triplet state (2) to the ground state (0) to represent phosphorescence. A "vertical" relaxation transition icon should be chosen from the icons bar on the left (see below).

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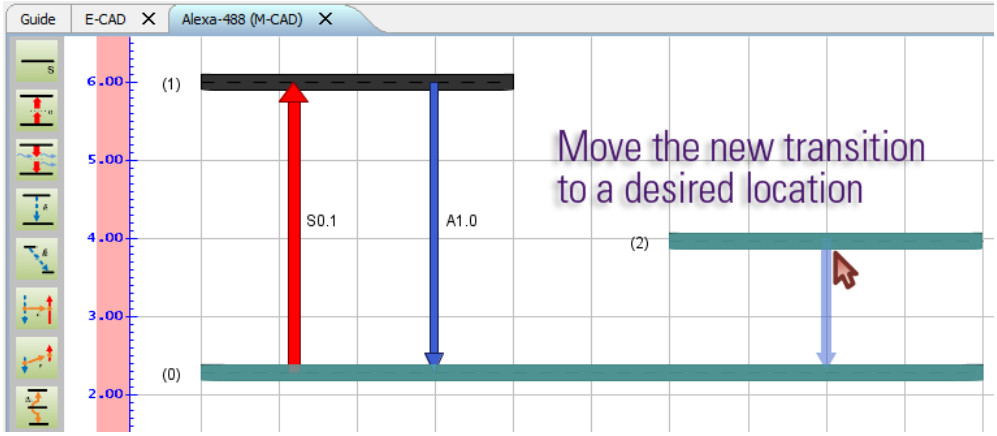
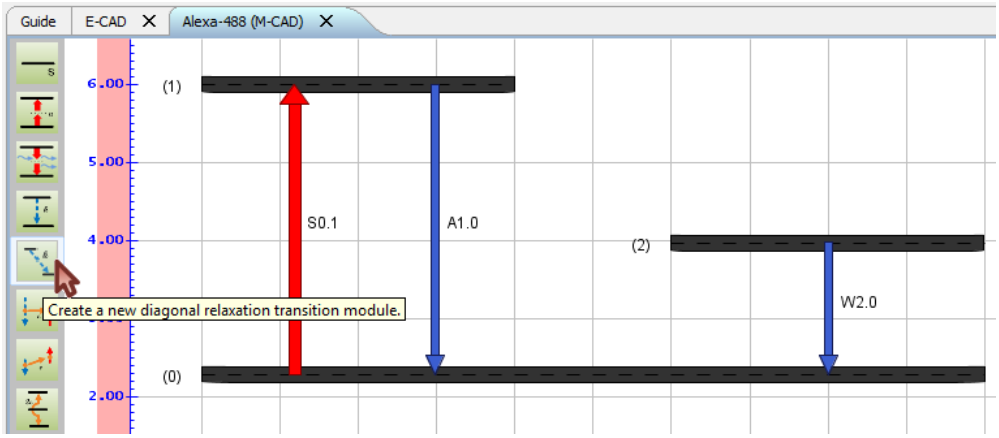
The new phosphorescence transition is placed in the same manner, locked between energy levels (0) and (2).



Now, one can add the inter-system crossing transition which represents the relaxation from singlet excited state (1) to the triplet state (2). The "diagonal" relaxation transition icon should be used for this case.



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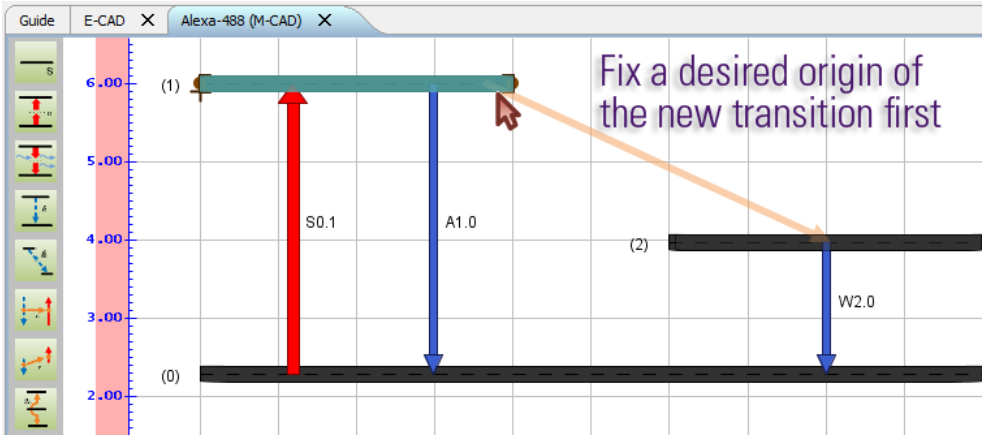
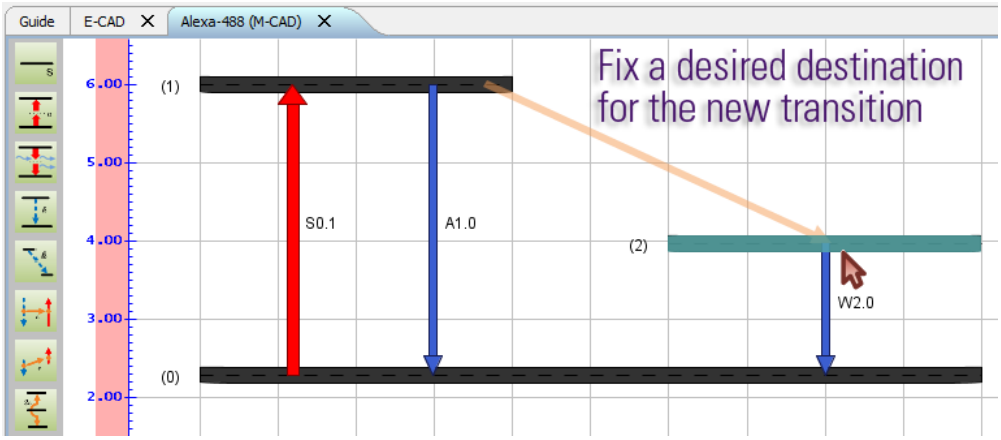
The newly created transition should be locked between levels (0) and (2) in two steps: first mouse click will fix the origin of the transition,



and the second mouse click will fix its destination.



SimphoSOFT will automatically adjust the numerical algorithm by adding new transitions to the rate equations and the laser propagation equation. Its Equation panel shows the changes immediately.

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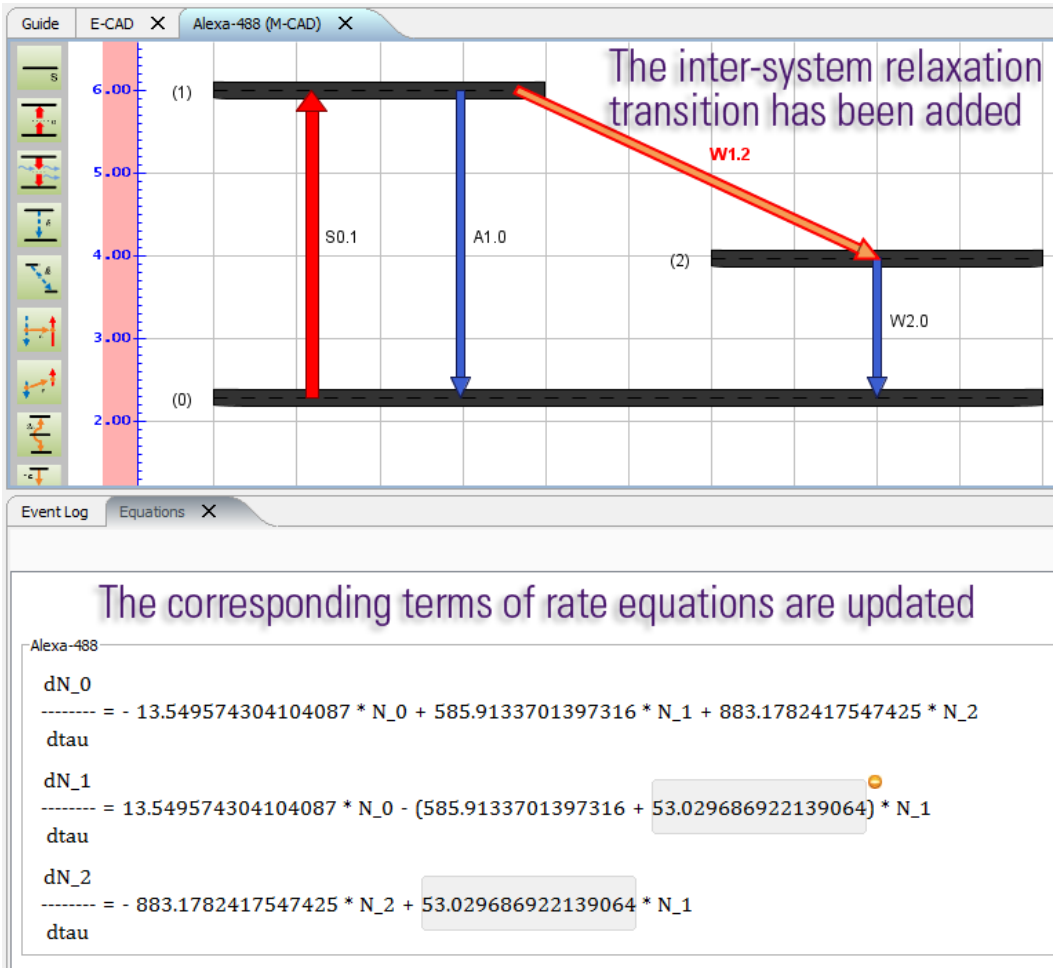

One can now model laser interaction with fluorescent probe molecules within SimphoSOFT and calculate fluorescence and phosphorescence output signals.

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