Guide to exporting commonly requested data from within the LAS X FALCON/SMD Wizard

Purpose:

FALCON users frequently request the ability to export lifetime data in one of two common formats for accessible interpretation and analysis.

The first format is a pixel-by-pixel matrix of mean lifetime, with mean lifetime data for each XY pixel location mapped to a corresponding position within the representative matrix. This data is commonly requested for import into MATLAB, ImageJ, or Excel for in-depth quantitative analysis.

The second format is similar to our Fast FLIM map, but instead, each pixel is color coded to fit average lifetime data following a pixel-by-pixel fit of the data. This format is useful for quick, rough qualitative visual analysis and decision making, and is similar to exported formats available on competitors FLIM systems.

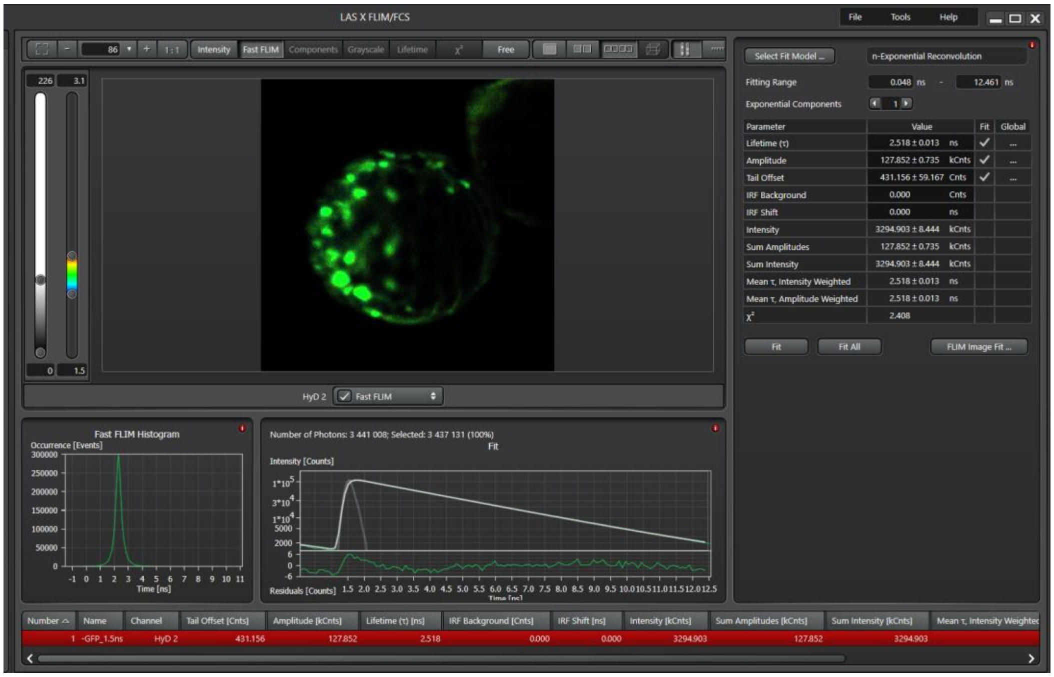
The guide below is separated into two parts, highlighting how to accomplish each of these tasks.

Part A: Generating an “average lifetime matrix”

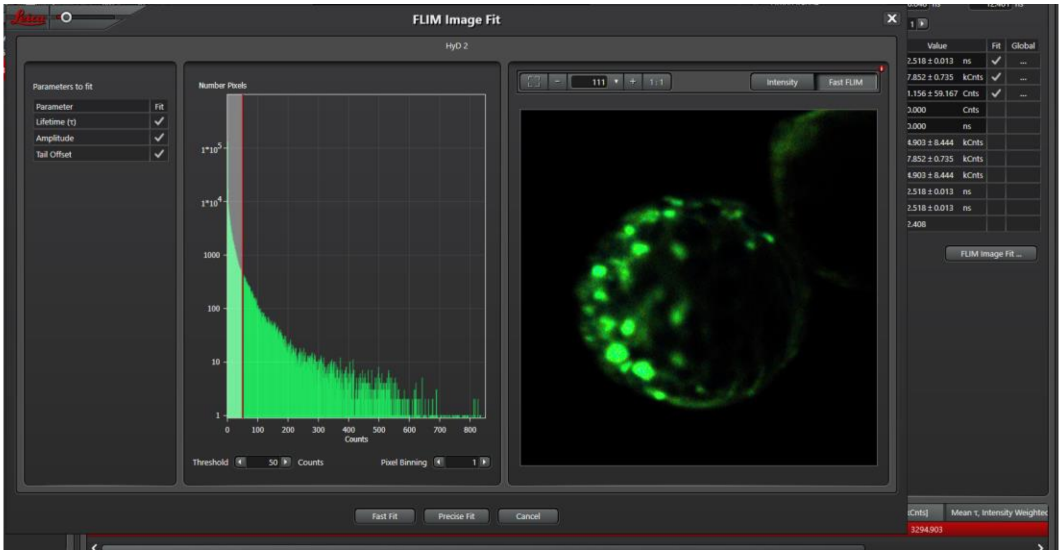
Using the steps detailed in this guide, you can generate a 16-bit ImageJ TIFF containing a pixel-by-pixel matrix of fit lifetime data (Mean τ, Intensity or Amplitude Weighted) mapped to each XY position within an image.

In the resulting image format, a 16-bit monochrome ImageJ TIFF, the intensity (grey level) of any given pixel will directly represent the fit Mean τ for that pixel in picoseconds. (*e.g.* a measured pixel with an amplitude weighted mean τ of 1.765ns will be displayed as an intensity of 1765 on a 16-bit scale).

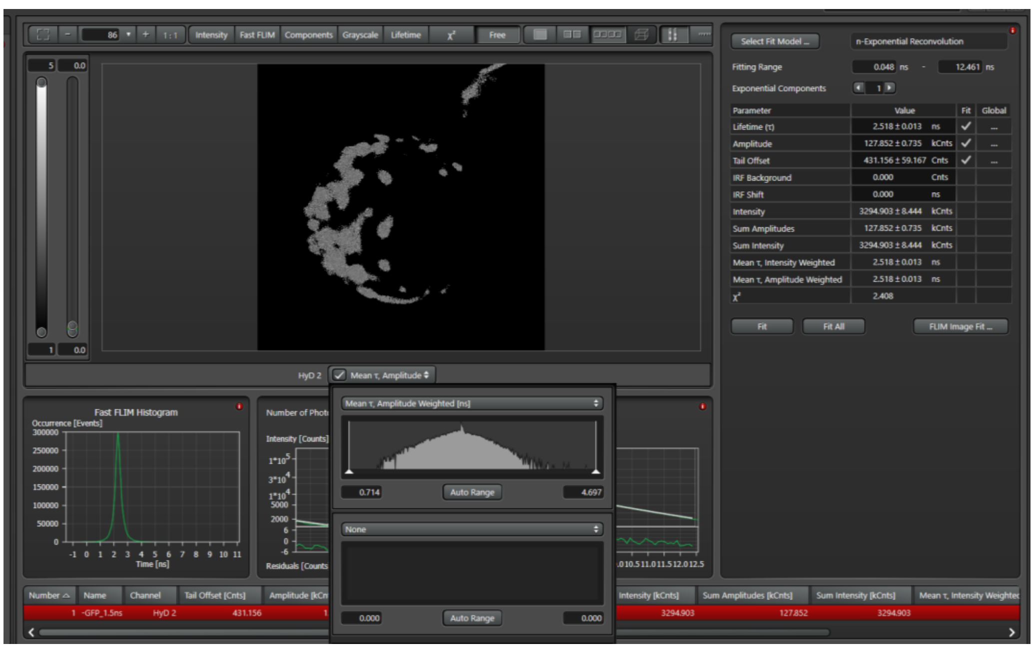
1) Perform an appropriate fit on the raw FLIM data using the fitting tool.



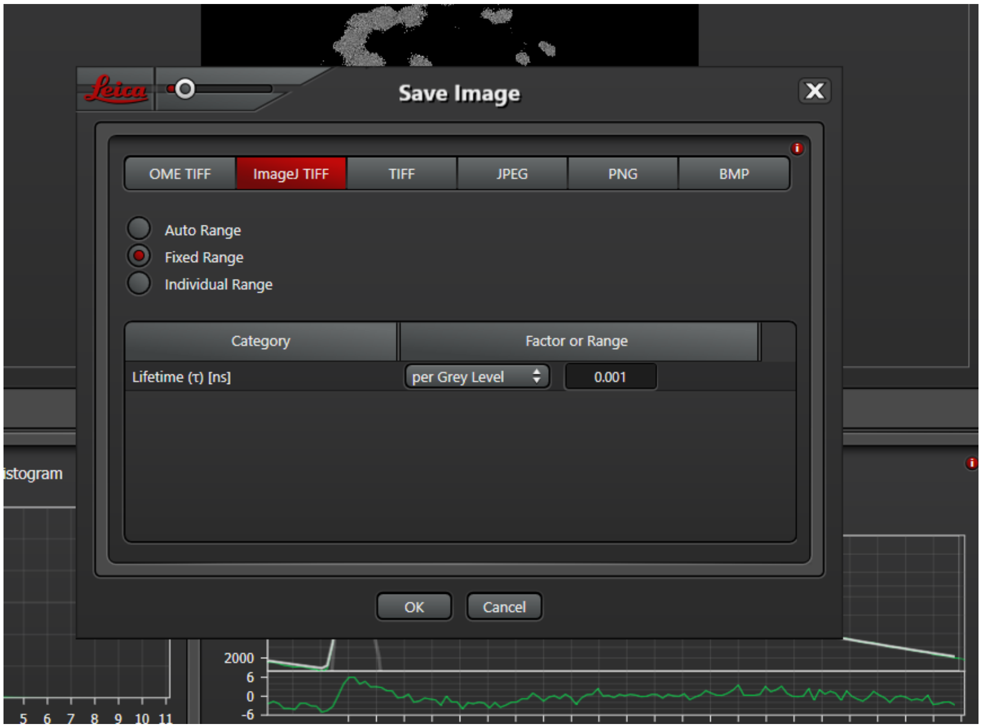
2) Perform a FLIM Image Fit. ***MAKE SURE TO THRESHOLD APPROPRIATELY TO AVOID LOW PHOTON-COUNT PIXELS/NOISY DATA***



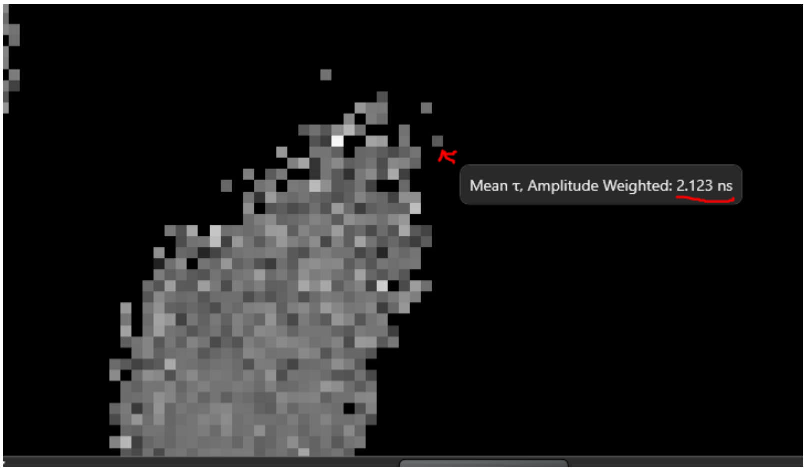
3) Go to the Free Viewer and map Mean τ (Intensity or Amplitude Weighted) to the first parameter (Grey Level). The second (color) parameter should be set to none



4) Right click on the image and choose “Export Raw Image”.
Choose ImageJ TIFF and set the range to 0.001ns per Grey Level.



5) The resulting exported image can be opened in ImageJ and pixel-by-pixel grey level values may be compared



6) The ImageJ Results Viewer (Image -> Transform -> Image to Results) help to view and export Mean τ in a simple pixel-indexed numerical format.

Data can be copied/pasted from here into excel for more detailed anlysis.

0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1980	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3071	2617	2594	0	0	0	2483	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2841	0	2648	2410	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2093	2460	2063	2924	3544	2503	0	0	0	2502	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1992	1658	4541	0	0	3258	3130	0	2578	0	0	2123	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3062	0	0	2532	2528	2204	0	2326	2378	2156	1884	2203	2523	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2708	3279	1531	2570	2859	2194	2322	2518	1919	2829	2740	2270	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	2698	0	2140	2609	0	0	1502	2553	2105	1489	2576	2874	2000	3329	1699	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	2339	0	0	2501	2674	0	2606	2543	3609	2954	2094	2169	2510	1727	0	0	2362	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	2537	1522	3131	2518	2373	2531	2306	2182	1957	2212	2756	2443	2333	1766	2952	2944	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	2961	0	2031	2164	1889	2060	2444	2007	3113	2408	1674	2177	2325	2248	2218	3674	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	2418	2703	2868	2277	2373	2478	2738	2620	2132	2945	2744	2675	2469	2859	3074	0	0	0	0	0
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0	0	0	0	0	2965	0	2344	2241	1297	1779	2269	2502	2574	2555	2691	2633	2102	1957	2554	2687	2497	2488	1812	2152	1296	2519	2343	2524	0	0	0	0
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0	0	0	0	0	2655	2206	2443	2117	2008	2517	2251	2523	2614	3153	2381	2607	2306	2950	2650	2736	2502	2330	3217	2328	2060	2411	2059	0	0	0	0	0
0	0	0	0	2396	2032	3171	2124	2110	3170	2602	1905	2689	2462	2701	2194	2223	2442	3319	2890	3325	2184	2539	2755	1693	3898	0	1873	3169	2428	0	0	0
0	0	0	0	2067	2843	2518	2377	2430	2065	2423	2442	2447	1923	2293	2011	2259	2428	1973	2379	2635	2575	2331	2537	2608	2318	2610	2954	2702	0	0	0	0
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0	0	3232	0	2489	3183	2532	2430	2052	2324	2518	2101	2701	2340	2852	2797	2638	2281	2536	2537	2533	2281	2110	2634	2540	2541	1685	0	0	0	0	0	0
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0	2888	0	2407	2938	2836	1982	2603	2850	2491	2609	2553	2418	2676	2444	2973	1939	2525	2493	2522	2402	2054	2006	2502	1850	0	0	0	0	0	0	0	0
0	0	2425	2248	2541	2527	2749	2311	2552	2706	2525	2522	2631	2557	2389	2708	2357	2386	2518	2603	3054	2908	2456	2157	2502	0	0	0	0	0	0	0	0
0	0	2409	2427	2538	2947	2775	2416	2648	2277	2528	2194	2487	2387	2196	2224	2818	2677	2713	2737	2238	2486	2482	2806	2835	0	0	0	0	0	0	0	0
0	2297	2619	1615	2353	3014	2307	2570	2467	2509	2760	2802	2534	2510	2269	2175	2685	2324	2550	2552	2518	2421	0	2009	2724	0	0	0	0	0	0	0	0
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0	0	2070	1857	2472	1863	2491	2555	2350	2573	2657	2587	2616	2632	2204	2422	2514	2310	3289	2284	2388	2532	2154	0	0	0	0	0	0	0	0	0	0
0	0	0	2815	2440	2522	2846	2614	3106	2614	2637	2881	2206	2830	2784	2396	3369	2383	2540	2394	2623	2474	2431	0	0	0	0	0	0	0	0	0	0
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0	0	0	2781	2568	2474	2859	2342	2802	2375	1813	3014	2976	2701	2428	2911	2331	2532	2760	2499	2335	2263	0	0	0	0	0	0	0	0	0	0	0
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0	0	0	0	2326	2270	1843	2264	3292	2799	2680	2404	2537	2684	2180	2621	2659	3111	2674	3067	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	2993	2202	2562	2511	2095	3220	2963	2576	2842	2248	2964	2803	2600	2085	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	1580	2367	2538	2538	2683	1677	2518	2347	1405	2649	3002	1960	0	2301	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	2383	0	3487	2634	2543	2256	2562	0	2518	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Part B: Generating a B&H style color-coded average lifetime map

The purpose of this technique is to generate a separate color-coded image that may be useful for more immediate qualitative visual analysis, or may reflect data exported in a specific manner that the customer may request.

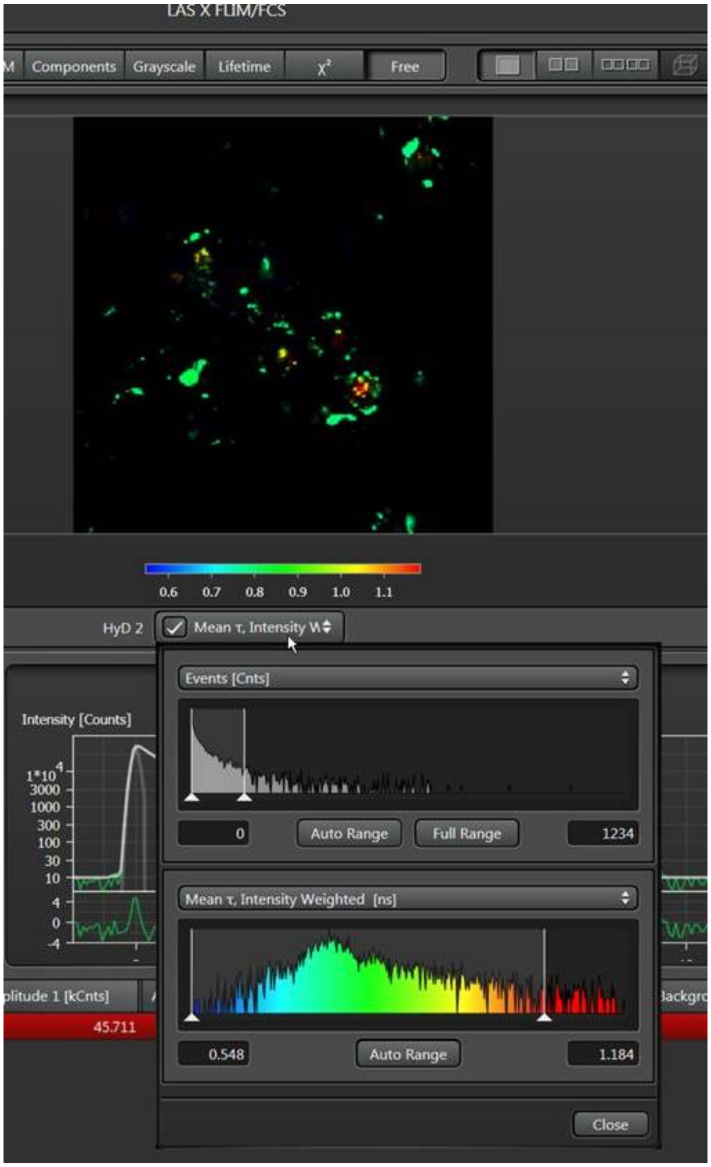
This process generates a standard RGB TIFF with a separate file for the average lifetime lookup table or “lifetime scale bar”

In this image, average lifetime of each pixel (mean τ) is mapped to color from cool to warm (shortest lifetimes will be blue, longest will be red).

Fluorescence intensity at any given pixel (# of counts) is normalized to higher pixel intensity within the RGB tiff. However, note that in this format, quantitative data regarding number of events is not easily distillable, and as most people have difficulty distinguishing different intensities with high color variation by eye, caution should be taken when attempting to make any intensity-based judgement using the exported image.

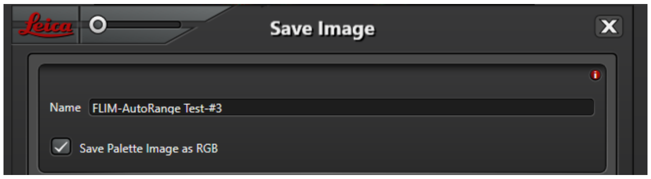
1) Perform steps #1 and #2 from Part A of this guide (perform an appropriate fit on the lifetime decay curve, then perform a FLIM image fit)

2) Within the free viewer, set the first parameter (grey level) to “Events [CNTs], and set the second parameter (color) to Mean τ, intensity or amplitude weighted

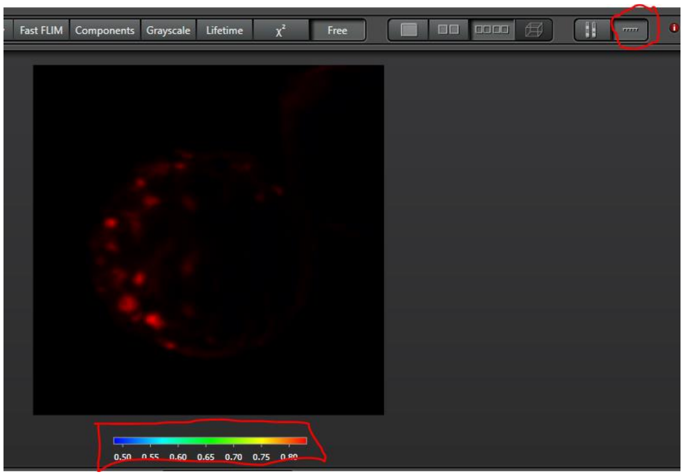


3) Right click on the image and choose “Export Image” and export to a suitable directory.

3a) Alternatively, the image may be saved to the LIF project using the “Save Image” button on the FLIM TAB. Here, you will want to choose “Save Palette Image as RGB” option



4) There is, presently, no method for exporting the color look-up-table map for fluorescence lifetime (*e.g.* the so-named “lifetime scale bar”) – the current workaround is to choose the “display lifetime scale bar” feature within the LAS X GUI, and to use the Windows “Snipping Tool” to capture a screenshot of said scale bar and save it along with the exported image



Field Observations & Cautionary Notes Regarding this Technique

Part A: Pixel-by-pixel matrix of average lifetime

1) The image to be analyzed will require both curve fitting and image fitting prior to image export. At present, Fast FLIM data cannot be exported in this way either before or after fitting.

   Other parameters like χ^2 also cannot be mapped to intensity in an exported image presently (as of SMD wizard version 3.55).

2) Note that, in the exported image, there is <u>no information corresponding to pixel intensity</u> contained within the exported image. It is, for all intents and purposes, more a lifetime matrix than an image

   However, the correlation between pixel intensity (grey value) in an image and signal intensity is very ingrained, and even experienced microscopists and confocal users may initially have difficulty understanding lifetime mapped as intensity in the image.

   It is, therefore, suggested to only utilize this technique when a user requests this sort of information specifically, or when it is specifically necessary for some deeper analysis, and to have a practiced and complete explanation of what the numbers mean.

   This technique can be helpful if a customer claims we do not sufficiently granular lifetime data, (e.g. pixel level data) for our images.

   It may also help to display the image results view immediately to the customer as opposed to the raw image, as it is more approachable.

3) Given the range in fluorescent lifetimes generally characterized on the system, most samples will not use much of the exported 16-bit image scale (*e.g.* a pixel with an average lifetime of 6.0 ns will be mapped to an intensity of only 6000 on a scale of 0 – 65535 using this method).

   As a result, the exported image is likely to be very dim, and have very little dynamic range (it may even look like a binary image when opened in ImageJ).

   This is another reason that immediately demonstrating the "results view" in ImageJ and the raw numbers is often more helpful than focusing on the image itself.

4) Because there is no information contained within the image on either # of photon counts and/or χ^2 contained within the exported image it is impossible to use only the exported matrix to make a judgement call about the quality of fit or accuracy of the mean lifetime at any given pixel location in the matrix. (*i.e.* If thresholding is not applied, average lifetime will be shown for a pixel where one photon is counted in the same way that it's shown for a pixel where 1000 photons are counted).

   Because of this, it is critical to properly threshold when curve fitting and image fitting in order to mitigate the effects of noise in the data, and avoid mapping pixels where an insufficient number of photons were counted to provide an accurate average lifetime

Part B: B&H Style Color Coded Lifetime Map

1) As mentioned above, intensity information is preserved in this image format and will be accurate relative to the image scale (*e.g.* higher counts will be brighter than lower counts). However, extracting precise information regarding either the number of counts, or the exact average lifetime at any given pixel from the exported image is non-trivial.

   If a customer requests this sort of image, we can give it to them, but if they then ask how to analyze it in detail they should be pointed to the raw data within the LAS X SMD tool.

2) Presently, export of the lifetime scale bar is not ideal. If you know a customer will need to export their data in this format, it is best to set up a specific folder for each dataset and place both the exported RGB TIFF and the snapshot of the "lifetime scale bar" within the folder to make sure they stay together. Otherwise, the information may be lost and the image will not be interpretable.

   Always point out that the process can be repeated if the raw LIF data is maintained.