**Phagosomal acidification measurements with confocal microscope (Leica SP5)**

1. Conjugate a new batch of Zymosan-pHrodoRed/RhodamineGreen. RhodamineGreen is needed for automated tracking the Zymosan particles and performing ratio measuments of pH.
2. For each batch of Zymosan-pHrodoRed/RhodamineGreen laser confocal microscope parameters (like laser power and PMT gain) should be set in order to avoid signal saturation and utilizing maximal dynamic range of the pH indicator. That can be done by imaging Zymosan particles at minimal physiological phagosomal pH (~4.5)and neutral pH (~7.3).

Parameters to set:

* Choose sequential imaging in order to avoid channels crosstalk
* For imaging **pHrodo Red** signal use:

Laser line: **561nm** Laser power:

PMT emission range: **580nm - 620nm**

Adjust Laser power, PMT Gain and Offset at pH 4.5 just below signal saturation. Don’t use laser power more than 20% in order to avoid cell damage.

PMT#: Gain: Offset:

* For imaging **Rhodamine Green** signal use:

Laser line: **488nm** Laser power:

PMT emission range: **510nm – 550nm**

PMT#: Gain: Offset:

In addition to channels for recording pHrodo Red and RhodamineGreen use a channel for recording DIC image.

**Always use same imaging settings for same Zymosan batch and experiment type.**

1. Preheat microscope to +37C at least for 15min in order to stabilize thermal drift.
2. Start LAS software
3. Initialize XY-stage for multi-position imaging
4. In microscope Settings choose 12 bits and line averaging, set Argonne laser power to 20%
5. Set imaging parameters (see above) either manually or load a previously saved sequence or apply setting from old file.
6. Use objective 63.0x1.40 OIL
7. Use 1024x1024 or 512x512 pixels resolution
8. Use constant Zoom level (usually 3x)
9. Choose multi-positions imaging
10. Set different positions for imaging cells.
11. Set Z-stack levels (first bottom level, than upper level). Use Z-stack steps size - 1µm.
12. Choose XYZT for time lapse imaging.
13. Set imaging interval – 1min and duration 30 min or more.
14. Add Zymosan particles to cells. Wait ~1 min for Zymosan to settle down and start imaging.
15. During imaging make sure that focal plane doesn’t drift.
16. After imaging stops, safe data on server.