How to check the overlap of different color confocal volumes

Because both lasers are fed through the same fiber, there cannot be any optical beam displacement. According to my records, your system has the latest apochromatic fiber outcoupler, therefore there is practically nothing you can improve: lasers mounted in the LCU are always well overlapped. This is a simple consequence of the MicroTime 200 design.

However, in theory a confocal volume overlap of different colors will never be perfect. The reason is that the degree of chromatic correction of the objective has its own limits. The best results are obtained by the latest UPLSApo (UltraPlan Super Apochromat) objectives.

With a PIE capable setup, you can evaluate the overlap by imaging a single, sub-resolution, multicolor particle, for example a 100 nm diameter TetraSpeck bead, using both lasers simultaneously.

You need an appropriate dual band major dichroic and PIE excitation with the Sepia. In this case there is no need for detection color splitting. One detector is sufficient. Use a single long pass filter, target and image a single bead. Of course, you have to set the laser intensities so that both laser excitations contribute with approximately half of the final detected sum count rate.

In SymPhoTime64, calculate two images: one for each PIE time window, that is, one for each excitation laser. (It is easy with the SymPhoTime64, use the graphical tool for setting the time-gates.) By comparing the two images, you should see no significant displacement.