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Rizal,

It is good to hear that the TIRF objective is giving better illumination. The problem with getting TIRF to work may be, as you say, that the spot size is too big. One could try a pinhole to see if that would work. A pinhole will reduce the total light delivered to the sample. An alternative would be to use a beam expander or reducer.

You could try making a beam reducer by taking two lenses having different focal lengths and spacing them apart so that parallel incoming light beams also exit in a parallel manor. Another option would be to buy a beam expander and run light through it in reverse. Edmund Optics and Thorlabs both offer a selection of beam expanders.

I expect that the alignment for TIRF will be a bit tricky. If the laser beam is not entering the microscope objective vertically but is coming in at some angle, depending on which side of the objective is illuminated, the light may not come out of the objective at a shallow enough angle to reach the critical angle. The sample will also have to be thin enough so that the microscope objective can focus through one cover slip onto the other cover slip.

Attached is a sketch of a test fixture that might help in getting TIRF to work. It would allow you to test the TIRF objective without using the microscope. It consists of a well that is glued to a cover slip using a water resistant glue like Silicon glue. If you took one of the plastic cuvetts that you use for quantitating and cut off the tapered end it would work as a well. This would let you monitor the angle at which the transmitted laser light comes out. One would hope to see the angle θ go to zero as the laser beam is moved toward the edge of the objective lens. One could also monitor what the reflected beam is doing. TIRF will have been achieved when θ has gone to zero and all the light is reflected light. This should let you quickly see what is going on without also having to adjust and focus the microscope. Once you get this to work, you can try getting the entire system to work. When working with the test fixture be careful to insure that the laser beam does not directly enter your eye. It would be a good idea attenuate the laser light as much as possible consistent with having enough light left to follow the beam paths.

Best regards,

Bernie Yurke