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Fluorescence correlation spectroscopy (FCS) is a powerful experimental technique that in recent years has found numerous applications for studying biological phenomena. In this article, we scrutinize one of these applications, namely, FCS as a technique for studying leakage of fluorescent molecules from large unilamellar lipid vesicles. Specifically, we derive the mathematical framework required for using FCS to quantify leakage of fluorescent molecules from large unilamellar lipid vesicles, and we describe the appropriate methodology for successful completion of FCS experiments. By use of this methodology, we show that FCS can be used to accurately quantify leakage of fluorescent molecules from large unilamellar lipid vesicles, including leakage of fluorescent molecules of different sizes. To demonstrate the applicability of FCS, we have investigated the antimicrobial peptide mastoparan X. We show that mastoparan X forms transient transmembrane pores in POPC/POPG (3:1) vesicles, resulting in size-dependent leakage of molecules from the vesicles. We conclude the paper by discussing some of the advantages and limitations of FCS as compared to other existing methods to measure leakage from large unilamellar lipid vesicles.

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