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Quantitation of secreted proteins using mCherry fusion constructs and a fluorescent microplate reader

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ABSTRACT

Traditional assays for secreted proteins include methods such as Western blot and enzyme-linked immunosorbent assay (ELISA) detection of the protein in the cell culture medium. We describe a method for the detection of a secreted protein based on fluorescent measurement of an mCherry fusion reporter. This microplate reader-based mCherry fluorescence detection method has a wide dynamic range of 4.5 orders of magnitude and a sensitivity that allows detection of 1 to 2 fmol fusion protein. Comparison with the Western blot detection method indicated greater linearity, wider dynamic range, and a similar lower detection threshold for the microplate-based fluorescent detection assay of secreted fusion proteins. An mCherry fusion protein of matrix metalloproteinase-9 (MMP-9), a secreted glycoprotein, was created and expressed by transfection of human embryonic kidney (HEK) 293 cells. The cell culture medium was assayed for the presence of the fluorescent signal up to 32 h after transfection. The secreted MMP-9–mCherry fusion protein was detected 6 h after transfection with a linear increase in signal intensity over time. Treatment with chloroquine, a drug known to inhibit the secretion of many proteins, abolished the MMP-9–mCherry secretion, demonstrating the utility of this method in a biological experiment.

Protein secretion from cells is a fundamental process of great importance in normal biology and pathobiology. Establishment of the secreted protein discovery initiative [1] and a web-based secreted protein database [2] has provided much new information on defining the secretome. In addition, the recent availability of many new bioinformatics tools [3] has helped in the interpretation of the biological significance of the secretome. Exogenous expression of a fusion protein between a secreted protein of interest and a fluorescent reporter such as green fluorescent protein (GFP)[1] has been used extensively to characterize the subcellular localization and trafficking of secreted proteins since the earliest days of the discovery of the fluorescent reporter (reviewed by Presley in Ref. [4]). Incorporating methods such as time lapse imaging, fluorescent recovery after photobleaching, and Förster resonance energy transfer has extended the capability of fluorescent reporter tagging to accurately report on dynamic protein movement and protein–protein interaction within a specific subcellular compartment involved in secretion. A recent report confirmed that for a majority of proteins, the addition of a fluorescent reporter does not alter the subcellular localization compared with the untagged proteins detected by immunofluorescence imaging against the native proteins [5]. Despite the use of a fluorescent reporter for intracellular spatiotemporal characterization, the use of fluorescent reporters to detect secreted proteins once the protein leaves the cells has not been explored. Experimentally, the detection of secreted and non-secreted proteins retained within cells is still accomplished by a Western blot or an enzyme-linked immunosorbent assay (ELISA) of the secreted protein in the cell culture medium, requiring considerable time and cost. Here we describe a simple method extending the utility of fluorescent reporter-tagged fusion protein to enable detection of the secreted proteins in the cell culture medium and the non-secreted retained protein within the cell using a fluorescent microplate reader.

Materials and methods

Reagents

Chloroquine diphosphate (cat. no. C6628) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Purified mCherry (cat. no.

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[1] *Abbreviations used:* GFP, green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; MMP-9, matrix metalloproteinase-9; HEK, human embryonic kidney; cDNA, complementary DNA; PCR, polymerase chain reaction; eGFP, enhanced GFP; ss–eGFP, signal sequence eGFP; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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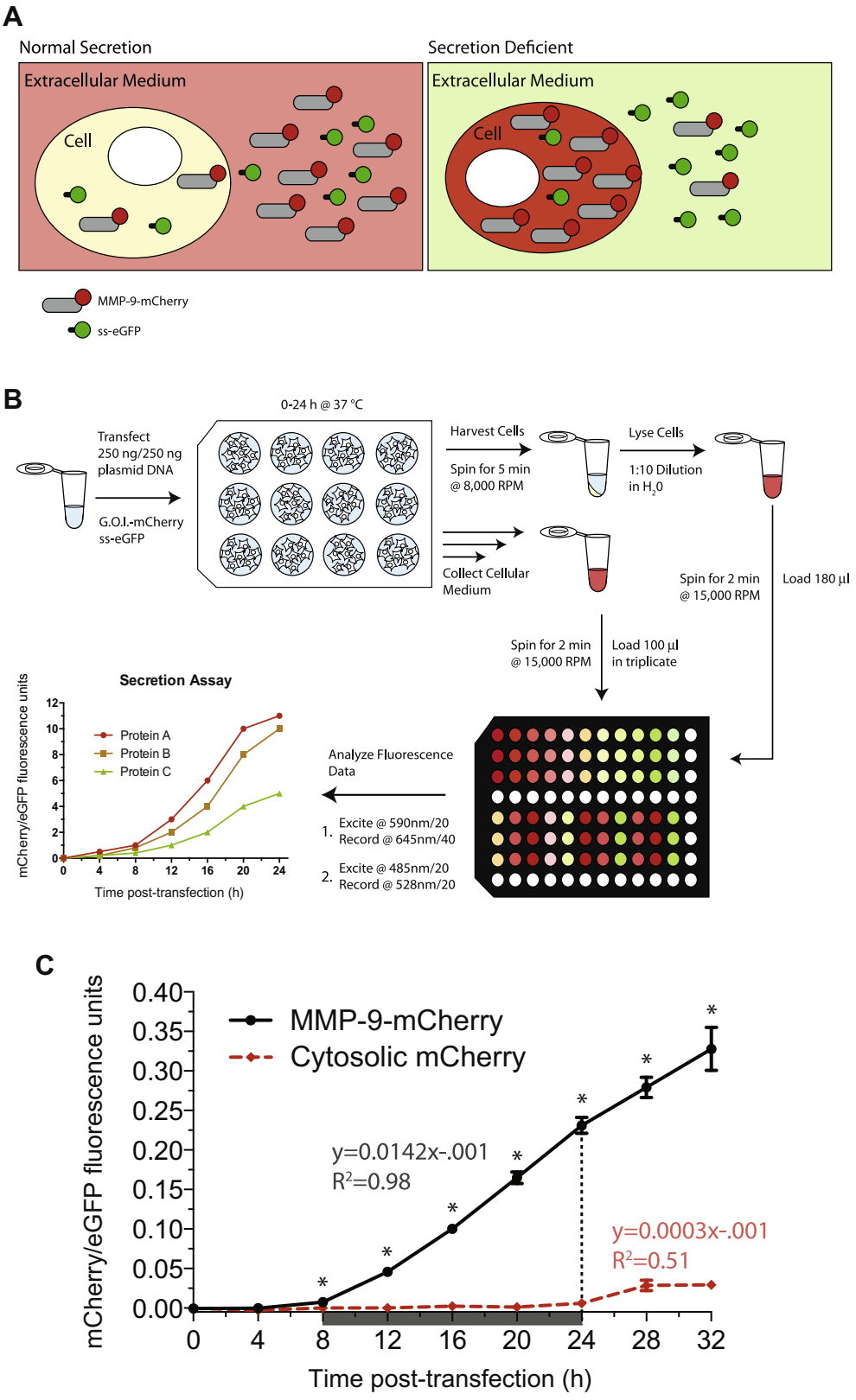

Fig.1. mCherry-tagged protein secretion assay. (A) Cartoon outline of the assay. The gene of interest (MMP-9) is subcloned upstream of mCherry and transfected into cells along with a plasmid encoding the secretable form of GFP (ss–eGFP). The fluorescent reporter read-outs are used to quantify intracellular protein or secreted protein in the extracellular medium. (B) Experimental flow diagram for the 12-well secretion assay for a particular gene of interest (G.O.I.) fused to mCherry. Prototypical data are reported as ratios of mCherry/eGFP as a function of time for three prototypical proteins (A–C) indicating fast (protein A, red line), intermediate (protein B, orange line), and slow (protein C, green line) rates of secretion. (C) Cells were transfected with MMP-9–mCherry and ss–eGFP, and the normalized fluorescent signals (black line) in the culture medium was followed over time. A control with cytosolic-restricted mCherry (red dashed line) allowed detection of dead cells releasing the fluorophore into the medium. The rate of secretion (arbitrary units) and R^2 value were calculated from the linear region of data from 8 to 24 h post-transfection (gray boxed area). Student t test; $^*P < 0.05$; means ± standard errors ($n = 3$).