Supplementary material legends:

Supplementary Software 1: Microscopy software to manually acquire FCS-calibration data.

Contains the VBA macro *FCSRunner.lvb* for acquiring imaging and FCS data to later compute the FCS calibration parameters. The macro runs with ZEN black edition (version ≥ 2010) on Zeiss LSM microscopes. Please read the documentation located in the *wiki* directory or the Wiki at *https://git.embl.de/grp-ellenberg/fcsrunner/wikis* for detailed information on how to operate the software. In order to keep track of possible software updates, the user can clone the package from the primary public software repository using the following *git* command: *git clone https://git.embl.de/grp-ellenberg/fcsrunner.git.*

Supplementary Software 2: Microscopy software to automatically acquire FCS-calibration data.

Contains the VBA macro *MyPiC.lvb.* The macro runs with ZEN black edition (version ≥ 2010) on Zeiss LSM microscopes. *MyPiC* is used to perform complex imaging and FCS workflows with the option to integrate online image analysis (see **Supplementary software 3**). The latter feature can be used to automatically acquire a large set of imaging and FCS data for the FCS calibration curve. Please read the documentation located in the *wiki* directory or the Wiki at *https://git.embl.de/grp-ellenberg/mypic/wikis* for detailed information on how to install and operate the software. In order to keep track of possible software updates, the user can clone the package from the primary public software repository using the following *git* command: *git clone https://git.embl.de/grp-ellenberg/mypic.git.*

Supplementary Software 3: Analysis software for automatic acquisition of FCS-calibration data.

FiJi plugin *Automated_FCS_.py* and related software. The plugin allows performing online image analysis for images acquired using *MyPiC*. The plugin performs intensity based segmentation in combination with a watershed step to separate merging objects. The segmented objects are then used to define new coordinates for imaging and/or fluorescence correlation measurements. Please read the documentation located in the *wiki* directory or the documentation at *https://git.embl.de/grp-ellenberg/adaptive_feedback_mic_fiji/wikis* for detailed information on how to install and operate the software. In order to keep track of possible software updates, the user can clone the package from the primary public software

repository using the following git command: *git clone https://git.embl.de/grp-ellenberg/adaptive_feedback_mic_fiji.git.*

Supplementary Software 4: Analysis software to process the FCS and imaging data.

Analysis workflow for FCS-calibrated imaging. The software package contains *MATLAB*-, *R*-, and *FiJi*-based tools to extract fluorescence intensities, compute FCS calibration parameters and convert the fluorescence intensities to physical quantities. The package includes:

- *FCSImageBrowser* (in folder *fiji*) is a *FiJi* script to browse through the acquired images and extract the fluorescence intensities at the FCS measurement points.
- *FCSFitM* (in folder *matlab/FCSFitM*) is a *MATLAB* tool to batch fit the correlation data, compute the effective volume and the concentrations. For convenience a compiled Windows installer is included (*FCSFitM_web.exe*). The compiled version does not require a *MATLAB* license.
- *FCSCalibration* (in folder *R/FCSCalibration*) is a *shiny R* application to interactively perform quality control of the data and compute FCS calibration parameters. For convenience we include a packed version for Windows.
- *FCSCalibrateImage* (in folder *fiji*) is a *FiJi* script to convert fluorescence intensities to concentration and protein numbers.

Please read the documentation located in the *wiki* directory or the Wiki at *https://git.embl.de/grp-ellenberg/fcsanalyze/wikis* for detailed information on how to install and operate the different elements of the software package. In order to keep track of possible software updates, the user can clone the package (without the compiled versions) from the primary public software repository using the following *git* command: *git clone https://git.embl.de/grp-ellenberg/fcsAnalyze.git.*