Chapter 3

Intracellular Dpp morphogen transport studied with Particle Image Cross Correlation Spectroscopy (PICCS)

Morphogens control pattern formation by forming concentration gradients. Gradients are formed by a combination of diffusion and degradation. Recently the effective diffusion and degradation rates of Dpp have been measured. Diffusion and degradation are determined by intracellular trafficking of morphogens: i.e. endocytosis, recycling, and degradation in the lysosomal pathway. However, these trafficking rates have not yet been measured. We followed the transport of the morphogen Decapentaplegic (Dpp) in wing imaginal discs of fruit fly larvae. These experiments required the development of a new analysis method for two-color, single-object data: Particle Image Cross Correlation Spectroscopy (PICCS). With this method we were able to quantify the fraction of Dpp that is correlated with early endosomes. We found that early endosomes contain almost twice as much Dpp as compared to other endosomes. Further we determined the rates underlying Dpp transport among different endosomal compartments. These rates are essential for a complete description of the intracellular transport of Dpp. This novel method is generally applicable to a multitude of biological processes that involve multiple interaction partners and makes use of the superior positional accuracy that is obtained in single-object microscopy.

3.1 Introduction

The morphogen Decapentaplegic (Dpp) forms a gradient in the developing wing imaginal disc of the fruit fly *Drosophila melanogaster*, ultimately controlling patterning and growth of the tissue. Dpp originates from a stripe of Dpp-producing cells at the anterior-posterior compartment boundary [1] and is secreted to neighboring cells. A recent study [2] showed that a steady state monoexponential gradient in Dpp is formed. While the latter study successfully and quantitatively describes the gradient on the level of the whole tissue, it provides insufficient insight into the (sub)cellular mechanisms that underlies Dpp transport. Other experiments further suggest that Dpp is spread by three different mechanisms: diffusion in the extracellular matrix [3], receptor-mediated diffusion [4] and by intracellular transport [5], i.e. multiple endocytosis and subsequent recycling events into the extracellular matrix [6]. The extracellular diffusion and receptor-mediated transport are governing short-range spreading, while intracellular transport is essential for long-range spreading of Dpp in tissue [7]. In the study described here we further elucidate the subcellular mechanisms of intracellular Dpp transport. Three types of endosomes are involved in Dpp gradient formation by intracellular transport: early, late and recycling endosomes. By using fluorescent endosomal markers and a fluorescent Dpp fusion protein we measured the fraction of Dpp in early endosomes and thereby determined the intracellular trafficking rates of Dpp.

To accomplish this we developed a new analysis technique which quantifies the amount of correlation between two fluorescent species. In the past several techniques have been applied to this problem. In particular single-molecule fluorescence assays have been used successfully to quantify colocalization of interaction partners [8–10]. Single-molecule fluorescence techniques require only small amounts of fluorescent labels and contain information about positional correlations on sub-diffraction length scales [11]. However, the direct mapping between single-molecule signals from two different channels is prone to a systematic error: colocalization is typically defined by a distance threshold below which two signals are considered colocalized. Therefore *a priori* knowledge about the distribution of distances, about the positional error, and about
the experimentally unavoidable alignment mismatch between two channels is needed to find a proper threshold. Even without any real correlation this method will always yield a colocalization event due to accidental proximity of signals. This problem aggravates with increasing signal density. Hence, even with the highest spatial resolution, proximity is not the optimal readout for correlation.

Fluorescence Cross-Correlation Spectroscopy (FCCS) and Image Cross-Correlation Spectroscopy (ICCS) directly determine the cross-correlation between the two different color channels [12, 13] without the need for a threshold. However, both have restrictions in accessible time scales, and proper treatment of sometimes heterogeneous background signals is not straightforward.

Here we show how the advantages of ICCS and single-particle tracking can be combined in one analysis technique: Particle Image Cross-Correlation Spectroscopy (PICCS). This technique is largely based on Particle Image Correlation Spectroscopy (PICS) developed by us before [11]. PICCS uses high accuracy single-molecule / single-object position data, but instead of correlating the positions of the same molecular species at several points in time (as is done in PICS), PICCS correlates the positions of two molecular species at the same point in time in two separate channels. Those channels can be two colors, as discussed below, or any other molecular parameter that allows distinction of two species like fluorescence signal level, fluorescence lifetime or polarization. By PICCS a correlation fraction and a correlation length are retrieved on time scales down to 1 ms. Since the input data consists of the positions of individual molecules / particles the autofluorescent background or additional noise sources do not influence the measurement. For the same reason the method is not limited to the diffraction of light and the correlation length can therefore be determined with nanometer accuracy. Finally, PICCS permits for the analysis of subpopulations. As demonstrated below, it is possible, for instance, to determine the correlation fractions for subpopulations which differ in intensity and obtain additional information in this way.

In the following we will present a detailed analysis of intracellular Dpp transport, based on 1) a mathematical description of intracellular Dpp trafficking and 2) the analysis of experimental data with PICCS. This combination
allows us to determine the intracellular trafficking rates of Dpp.

### 3.2 Intracellular transport of Dpp

Figure 3.1 illustrates intracellular Dpp transport. After endocytosis, a Dpp-containing vesicle fuses with an early endosome. From the early endosome, Dpp is either transferred to a late endosome for degradation, or to a recycling endosome, where it will be exocytosed into the extracellular matrix. While we regularly observed more than one endosome of each type per cell, in the following we treat each type of endosome as a single entity without loss of generality in modeling the intracellular trafficking of Dpp. This simplification is reasonable since we assume that the in- and outflow of Dpp only depends on the Dpp concentration (i.e. it is a first order reaction). This assumption is later confirmed by the data. The wing imaginal disc consists of a 2D-array of cells. Since the gradient is one-dimensional, perpendicular to the line of Dpp-producing cells, we model the disc as a 1D-array of cells. Inflow and outflow of Dpp is described by first order rate equations for each type of endosome. This approach details the theoretical description of Dpp spreading as described before [14], focusing solely on the intracellular trafficking.

Early endosomes receive an inflow of Dpp by endocytosis, depending only on the extracellular Dpp concentration $C_{ex}$ with an inflow rate $k_{ex}$. If the outflow of Dpp from early endosomes occurs with the rate $k_{ea}$, the change in Dpp concentration in the early endosomes of cell $n$ ($C_{ea}^n$) is given by

$$\frac{dC_{ea}^n}{dt} = \frac{1}{2} k_{ex} C_{ex}^n + \frac{1}{2} k_{ex} C_{ex}^{n+1} - k_{ea} C_{ea}^n$$  \hspace{1cm} (3.1)$$

assuming that the transport of Dpp is non-directional [5]. Since in fig. 3.1 we have defined $C_{ex}^n$ to be on the left of cell $n$, the endocytosis term consists of a contribution from both $C_{ex}^n$ and $C_{ex}^{n+1}$, taking into account non-directionality. For the recycling endosome a similar equation is derived, in which a parameter $\varepsilon$ is introduced to describe the fraction of Dpp in early endosomes that is
3.2 Intracellular transport of Dpp

Figure 3.1: Intracellular transport of Dpp. After endocytosis Dpp is transported – via early endosomes – to either late or recycling endosomes. While Dpp in late endosomes is destined to be degraded, Dpp in recycling endosomes is eventually exocytosed into the extracellular matrix.
Intracellular Dpp transport studied with PICCS

transferred to recycling endosomes.

\[
\frac{dC_r^n}{dt} = \varepsilon k_{ea} C_{ea}^n - k_r C_r^n \tag{3.2}
\]

where \(C_r\) is the concentration of Dpp in recycling endosomes and \(k_r\) is the rate of Dpp outflow from recycling endosomes. For late endosomes we obtain:

\[
\frac{dC_i^n}{dt} = (1 - \varepsilon) k_{ea} C_{ea}^n - k_i C_i^n \tag{3.3}
\]

where \(C_i\) is the concentration of Dpp in late endosomes and \(k_i\) is the degradation rate. Finally the concentration of Dpp in the extracellular matrix \(C_{ex}\) depends only on the recycling rate \(k_i\) and the endocytosis rate \(k_{ex}\), taking into account the non-directionality of intracellular Dpp transport.

\[
\frac{dC_{ex}^n}{dt} = \frac{1}{2} k_r C_r^{n-1} + \frac{1}{2} k_r C_i^n - k_{ex} C_{ex}^n \tag{3.4}
\]

Solving eqs. (3.1) to (3.4) in a steady state [5, 15] gives the average Dpp concentration in each type of endosome in each cell. From the solution we derive the fraction \(f\) of endosomal Dpp that is contained in early endosomes:

\[
f = \frac{C_{ea}}{C_{tot,endo}} = \frac{1}{1 + \frac{\varepsilon k_{ea}}{k_r} + (1 - \varepsilon) \frac{k_{ea}}{k_i}} \tag{3.5}
\]

From the known decay length of the Dpp-gradient (\(\lambda = 7.7 \pm 2.1\) cells) \(\varepsilon = 0.996 \pm 0.002\), is derived [2]. A more detailed theoretical derivation of eq. (3.5) and the determination of \(\varepsilon\) is found in section 3.A.1. For the recycling endosome outflow rate \(k_r\) we further derive:

\[
k_r = \frac{\varepsilon f k_{ea} k_i}{(1 - f) k_i - f (1 - \varepsilon) k_{ea}} \tag{3.6}
\]

In previous experiments the degradation rate \(k_d\) for Dpp has been determined [2]. Before a Dpp molecule is being degraded in a lysosome [5] it has been
endocytosed, transported to an early endosome and consecutively to a late endosome, after which it finally goes to the lysosome. Hence the degradation rate \( k_d \) can be expressed in terms of the other rates:

\[
\frac{1}{k_d} = \frac{1}{k_{ex}} + \frac{1}{(1 - \epsilon)k_{ea}} + \frac{1}{k_l}
\]  

(3.7)

Combining eqs. (3.6) and (3.7) gives for \( k_r \):

\[
k_r = \frac{\epsilon f k_{ea} k_{ex} k_d}{k_{ex} k_d - f k_{ea} (1 - \epsilon) (k_{ex} - k_d)}
\]  

(3.8)

which also gives an upper limit to the outflow rate for the early endosome \( k_{ea} \):

\[
k_{ea} < \frac{k_{ex} k_d}{f (1 - \epsilon) (k_{ex} - k_d)}
\]  

(3.9)

3.3 Particle image cross-correlation spectroscopy

Particle image cross-correlation spectroscopy (PICCS) is a method to quantitatively determine the correlation between arbitrary, fluorescently labeled molecules. Here we present the basic idea behind the PICCS methodology and algorithm. The theoretical background is detailed in section 3.A.2 and the scaling of the errors of the method is discussed in section 3.A.4. We assume that two interaction partners are labeled with two spectrally distinguishable fluorophores. The interaction partners can be single molecules or extended objects. We further assume that their density is so low that they can be resolved individually and their position determined with a high positional accuracy [16–18]. For simplicity we will denote the signals coming from the two fluorophores by ‘YFP’ and ‘CFP’ without loss of generality. The task is to determine the correlation fraction of the interaction partners, i.e. to determine the fraction of CFP that colocalizes with YFP (or vice-versa). In what follow we will calculate the fraction of YFP signals which are correlated to a CFP signal.

The first step in the PICCS analysis is identical to existing single-molecule tracking methods [9, 10]: the position of YFP and CFP signals is determined
Figure 3.2: PICCS algorithm. For all YFP signals (solid circles) the number of CFP signals (open circles) are counted which fall into a circle of radius \( l \) from a YFP signal. The total number is subsequently divided by the number of YFP signals. By increasing \( l \) from 0 to \( l_{\text{max}} \) the correlation function \( C_{\text{cum}}(l) \) is constructed. The dashed line encloses the area in which the YFP signals are used for analysis. This area is separated from the edges of the image by \( l_{\text{max}} \) (\( l_{\text{max}} = 2 \mu m \) is taken). The signal positions were simulated with the following parameters: density of YFP signals \( c_{\text{YFP}} = 1 \mu m^{-2} \), correlation fraction \( \alpha = 0.5 \) (results in a density of CFP signals of \( c_{\text{CFP}} = 0.5 \mu m^{-2} \)), correlation length \( \sigma = 150 \) nm.

with sub-diffraction positional accuracy by fitting two-dimensional Gaussians to the fluorescence signals. Subsequently a cross correlation function \( C_{\text{cum}}(l) \) between the two channels is calculated with an ensemble approach. \( C_{\text{cum}}(l) \) is equal to the average number of CFP signals at time \( t + \Delta t \) which have a distance smaller than \( l \) to a certain YFP signal at time \( t \) (fig. 3.2). When both fluorophores are imaged at the same time \( \Delta t = 0 \) s.

To avoid edge effects, only those YFP signals are used which lie farther away from the edges of the image than a predefined maximal distance \( l_{\text{max}} \) (\( 0 < l < l_{\text{max}} \), dashed line in fig. 3.2).
As detailed in [11] and Sec. 3.A.2, this procedure results in a correlation function of the form

\[ C_{\text{cum}}(l) = \alpha P_{\text{cum}}(l) + c_{\text{CFP}} \cdot \pi l^2 \] (3.10)

if the uncorrelated CFP signals are distributed randomly with a uniform density \( c_{\text{CFP}} \). \( P_{\text{cum}}(l) \) is the cumulative probability to find a distance smaller than \( l \) between a YFP and a CFP signal which are correlated. \( \alpha \) is the correlation fraction, i.e. the fraction of YFP signals which are correlated to a CFP signal. \( \alpha = 1 \) if there is a corresponding CFP signal for any YFP signal and \( \alpha = 0 \) if CFP and YFP signals are completely uncorrelated. In the form presented so far, the algorithm requires a random, homogeneous distribution of CFP signals, which results in the term \( c_{\text{CFP}} \cdot \pi l^2 \) in eq. (3.10). To correct for a non-random distribution of CFP signals we can calculate the spatial correlation among CFP signals by regular Particle Image Correlation Spectroscopy (PICS), see [11] and section 3.A.2.

Figure 3.3 shows an example for an experimentally determined correlation function. A wing imaginal disc of a fruit fly larva expressing Dpp-YFP and Rab5-CFP was imaged. Rab5 is a marker for early endosomes [19]. In total 28 \( z \)-stacks in both channels were taken. The endosome positions for each image in a \( z \)-stack were projected into one plane resulting in 28 YFP-CFP image pairs. Figure 3.3a,b show the first image stack for the YFP and CFP channel. The correlation function is shown in fig. 3.3c and the cumulative probability function \( P_{\text{cum}}(l) \) (eq. (3.12)) is shown in fig. 3.3d.

The density of CFP signals \( c_{\text{CFP}} \) and the correlation fraction \( \alpha \) were determined by fitting a straight line to the linear part of \( C_{\text{cum}}(l) \) plotted against \( l^2 \) (fig. 3.3c). The slope of this line gives \( \pi \cdot c_{\text{CFP}} \) while the offset is equal to \( \alpha \). After subtraction of the linear contribution and division by \( \alpha \), \( P_{\text{cum}}(l) \) remains.

If correlated signals were perfectly colocalized (both fluorescent molecules are at the same position in space), \( P_{\text{cum}}(l) \) would be given by

\[ P_{\text{cum}}(l) = 1 - \exp \left( -\frac{l^2}{2\sigma^2} \right) \] (3.11)

with the correlation length \( \sigma \). In any real experiment however, the positions of
Figure 3.3: Correlation fraction, signal density and correlation length from experimental data. A wing imaginal disc was imaged for 300 s using an alternating excitation method (described in section 3.4). Each image stack consists of 5 image planes (10 × 10 μm) separated by 0.7 μm in axial direction. Low frequency background was eliminated by applying a high-pass filter. 

**a)** Raw image stack from the Dpp-YFP channel (scale bar = 2 μm). 

**b)** Raw image stack from the Rab5-CFP channel.

**c)** Correlation function $C_{\text{cum}}(l)$ obtained by PICCS. Fitting to the linear part yielded a Dpp-YFP density of $c = 0.12 \pm 0.02$ endosomes · μm$^{-2}$ (solid line) and a correlation fraction of $\alpha_{e,\text{Dpp}} = 0.46 \pm 0.04$ (offset of the fitted line). 

**d)** $P_{\text{cum}}(l)$ which resulted from subtraction of the linear contribution from $C_{\text{cum}}(l)$ and division by $\alpha_{e,\text{Dpp}}$. The correlation lengths $\sigma_1$, $\sigma_2$ and the fraction $\beta$ were determined by fitting eq. (3.12) which gave $\sigma_1 = 71 \pm 17$ nm, $\sigma_2 = 161 \pm 34$ nm and $\beta = 0.44 \pm 0.17$, respectively. All errors were determined from simulations, see section 3.A.4.
particles cannot be exactly determined. This introduces an (apparent) correlation length, given by the finite positional accuracy $\sigma = \sqrt{2}\delta$, where $\delta$ is the one-dimensional positional accuracy for a fluorescent signal, see section 3.A.2. Any real correlation originating from the interaction between the studied objects adds to the correlation length given above.

In the experiments presented here, the signal intensity varies between signals and since the positional accuracy depends on the signal intensity, there is no well-defined overall positional accuracy. Therefore a modified version of eq. (3.11) including two effective correlation lengths ($\sigma_1$ and $\sigma_2$) is needed to describe the observed data.

$$P_{\text{cum}}(l) = \beta \left(1 - \exp \left( - \frac{l^2}{2\sigma_1^2} \right) \right) + (1 - \beta) \left(1 - \exp \left( - \frac{l^2}{2\sigma_2^2} \right) \right)$$

where $\beta$ is the fraction of data that has a correlation length $\sigma_1$. Adding more effective correlation lengths does not significantly improve the fit.

The method developed so far disregards signal intensities. We can therefore only determine the fractional amount of Dpp-containing early endosomes and not the fraction of Dpp molecules contained in early endosomes. Since there is no reason to assume that Dpp is homogenously distributed among the different types of endosomes, it is essential to include the intensity (which is a direct measure for the amount of Dpp molecules in an endosome) into the PICCS algorithm. We did this by weighting the occurrence of a YFP signal (Dpp-containing endosome) by its intensity. The average intensity of the correlated fraction was subsequently calculated by the offset of the correlation function and dividing the obtained value by that from the original unmodified correlation data (fig. 3.4).
3.4 Materials and Methods

The UAS-YFP-Dpp line was generated by using the existing UAS-GFP-Dpp vector [5] where GFP was replaced by YFP (Venus) [20]. dpp<sup>d8</sup>/dpp<sup>d12</sup>; dpp-Gal4/UAS-YFP-Dpp flies have an identical wing phenotype to the dpp<sup>d8</sup>/dpp<sup>d12</sup>; dpp-Gal4/UAS-GFP-Dpp flies [5]: they survive to adulthood and have normally patterned wings, although smaller in size (data not shown). For determination of early, recycling and late endosome distributions in wing imaginal discs we used the following genotypes: tub-CFP-Rab5, tub-YFP-Rab7 and tub-YFP-Rab11 [21].

To obtain wing imaginal discs, third instar larvae (tubulin-CFP-Rab; dpp-Gal4/UAS-YFP-Dpp) were dissected in Clone8 medium (Shields & Sang M3 Medium containing 2% Fetal Calf Serum, 2.5% Fly Extract, 12.5 IU Insulin/100 ml medium and 1X Penicillin/Streptomycin), after which the wing imaginal discs were mounted in a custom-made sample holder. Nail polish was used for sealing of the sample holder. The wing imaginal discs were imaged approximately 10 min after dissection. Samples were discarded 1 hour after dissection.

Imaging was done on a 3D wide-field fluorescence microscope as described previously [22]. To image a z-range of ~ 4 μm, image stacks were generated using a piezo-driven objective holder (Physik Instrumente, Karlsruhe, Germany) to move the objective in axial direction. Each image stack contained 5 image planes with Δz = 0.7 μm between each image plane. The time between image planes was 40 ms, during which the movement of the endosomes was negligible. Therefore each image stack was assumed to be acquired at one time point. Wing imaginal disc samples were excited by an Argon-ion laser (Coherent, Santa Clara CA, USA) at either 458 nm to excite Rab5-CFP or 514 nm to excite Dpp-YFP. An alternating excitation pattern was used to distinguish between the two fluorophores. The pattern consisted of 1 image stack which was excited at 458 nm and consecutively 10 image stacks were excited at 514 nm with a stack rate of 1 Hz. The fast switching between laser lines was done using an Acousto-Optic Tunable Filter (AA Opto-Electronic, Orsay, France). This pattern was chosen to minimize photobleaching of the CFP, since the amount of Rab5-CFP per endosome was lower than the amount of Dpp-YFP.
3.5 Results and discussion

In fig. 3.3 we showed for one wing imaginal disc that the correlated fraction of early Rab5-CFP labeled endosomes in the pool of all endosomes that contain Dpp-YFP in a wing imaginal disc is $\alpha_{e,Dpp} = 46 \pm 4\%$. Correction for a non-random distribution of CFP signals, see section 3.A.2, did not change

Figure 3.4: a) Correlation function for the correlation between Rab5-CFP and Dpp-YFP. Fitting a line to the linear part resulted in an offset at $l^2 = 0$ of $59 \pm 5\%$. Correction of the data using spatial correlations gives $\alpha_{Dpp,e} = 66 \pm 5\%$. b) Correlation function for the same data after weighting of the Dpp-YFP endosomes with their respective intensities. Fitting the linear part resulted in $\alpha_{Dpp,e} I = (5.2 \pm 0.4) \cdot 10^3$ counts, which gives an average Dpp-YFP signal of $\bar{I} = (8.9 \pm 1.0) \cdot 10^3$ counts for early endosomes that contain Dpp-YFP.

was done in the apical region of the cells since most of the endosomes are located there [2]. Dpp-producing cells (the source) were located by eye using a Mercury lamp (Zeiss, Oberkochen, Germany) for excitation. Using a motorized stage the center of the image was typically 20 μm displaced from the source, with an image area of 100 μm$^2$ showing approximately 17 cells in each experiment.
this result. Conversely the correlated fraction of Dpp-YFP containing endosomes in the pool of all Rab5-CFP labeled early endosomes was 59 ± 5% (see fig. 3.4a). After correction for the non-random distribution of CFP signals (see section 3.1.2) the latter value was finally increased to $\alpha_{\text{Dpp,e}} = 66 \pm 5\%$. Hence, two-thirds of the early endosomes contained Dpp, and about half of the Dpp-YFP containing endosomes were early endosomes.

To determine further whether Dpp molecules were homogeneously distributed over the different types of endosomes we used the ‘weighted’ PICCS method. Figure 3.4b shows the cross-correlation between Rab5-CFP labeled early endosomes and Dpp-YFP-containing endosomes weighted by the Dpp-YFP intensity. Fitting a straight line to the linear part gave a coefficient of $\alpha_{\text{Dpp,e}} \bar{I} = (5.2 \pm 0.4) \cdot 10^3$ counts. From the latter and our earlier result on $\alpha_{\text{Dpp,e}}$ (fig. 3.4a) we obtained the average intensity of Dpp in early endosomes to be $\bar{I} = (8.9 \pm 1.0) \cdot 10^3$ counts per endosome. The latter value, together with the knowledge of the total number of measured intensities of Dpp-YFP containing endosomes in the wing disc ($N = 735$ endosomes in 28 image stacks, data not shown) and the total fluorescence signal of Dpp-YFP in the wing disc ($I_{\text{tot}} = (4.9 \pm 0.8) \cdot 10^6$ counts in all image stacks, data not shown), the fraction of all Dpp-YFP molecules that was contained in early endosomes was

$$f = \frac{a_{\text{Dpp,e}} N \bar{I}}{I_{\text{tot}}} = 60 \pm 2\%.$$  

Hence, we found that early endosomes contained on average $n_{\text{Dpp,e}} = 1.8$ times as much Dpp-YFP as compared to late and/or recycling endosomes.

Segmentation with respect to the Dpp concentration in each endosome, as determined by the detected fluorescence signal, confirmed the latter finding. Analysis performed on the more intense half of the Dpp-YFP containing endosomes resulted in $\alpha_{e,\text{Dpp}} = 55 \pm 7\%$, and $\alpha_{e,\text{Dpp}} = 35 \pm 6\%$ for the less intense half of the endosomes (fig. 3.5a). Together our results showed that Dpp was not homogeneously distributed among the different types of endosomes. All results were independent on spatial data segmentation with respect to the cell’s distance to the Dpp-source.

As reported previously [23] the amount of Rab5 associated to endosomes fluctuates on the time scales of our experiments. To study the effect of Rab5
3.5 Results and discussion

Figure 3.5: Correlation fraction calculation $\alpha$ of early endosomes for segmented data sets. 

(a) Correlation fraction for Dpp-YFP-containing endosomes segmented according to Dpp concentration. For each image stack the Dpp-containing endosomes were divided in two halves, high intensity and low intensity. Applying PICCS on each subset shows that $\alpha_{e,Dpp} = 55 \pm 7\%$ of the high-intensity Dpp-containing endosomes were early endosomes, in comparison to $\alpha_{e,Dpp} = 35 \pm 6\%$ of the low-intensity Dpp-containing endosomes.

(b) $\alpha_{e,Dpp}$ calculated at different time points, using only 2 YFP-CFP image stacks for each data point. $\alpha_{e,Dpp}$ fluctuates around $47 \pm 14\%$, indicated by the dashed line. The large error bars are caused by the low amount of data in each point (i.e. only 2 CFP-YFP image stack pairs).

fluctuation on our measurements we analyzed the changes in correlation fraction over time. Instead of using the whole data set (28 YFP-CFP image stack pairs), we calculated the correlation fraction for data subsets containing 2 adjacent YFP-CFP image stacks (i.e. stack 1-2, stack 3-4, stack 5-6, etc.). In fig. 3.5b $\alpha_{e,Dpp}$ is plotted versus the time. $\alpha_{e,Dpp}$ fluctuates around $47\%$ with $\sigma = 14\%$, however no systematic trend on longer timescales was observed as has been reported for Rab5 fluctuations in early endosomes [23].

The results presented so far were obtained from one wing imaginal disc (17 cells in the field of view). We repeated the described experiments for two other wing imaginal discs (total number of cells=51, image centered at 20 μm
from the source), and found that $\alpha_{e,Dpp} = 38 \pm 2\%$ of Dpp-containing endosomes were early endosomes, and that early endosomes contain $f = 52 \pm 1\%$ of the endosomal Dpp with on average $n_{Dpp,e} = 1.9$ times as much Dpp in early endosomes compared to late and recycling endosomes.

All experiments were done in the apical 4.5 μm region of the cells. In order to draw conclusions about the kinetic parameters of intracellular Dpp trafficking, the complete apicobasal distribution of the different types of endosomes has to be considered. Both early and recycling endosomes are known to be located mainly in the apical region of epithelial cells [24, 25]. We confirmed this distribution for wing imaginal discs by measuring the apicobasal distribution of early, recycling and late endosomes over an axial range of 20 μm with Rab5-CFP, Rab11-YFP or Rab7-YFP, respectively. We found that 65% of the early endosomes, 41% of the recycling endosomes, and 62% of late endosomes are located in the most apical 4.5 μm of the disc, i.e. 51% of non-early endosomes were in the volume measured in our experiments, compared to 65% of the early endosomes. We corrected our results for this difference by calculating the real Dpp fraction $f$ using

$$f = \frac{\alpha_{e,Dpp} n_{Dpp,e} M_{non\text{-}early}}{\alpha_{e,Dpp} n_{Dpp,e} M_{non\text{-}early} + (1 - \alpha_{e,Dpp}) M_{early}}$$

in which $M_{early}$ and $M_{non\text{-}early}$ are the fractions of the total pool of early and non-early endosomes, respectively, which were in the measured volume. Taking into account that early endosomes contained 1.9 times as much Dpp as other endosomes we found a Dpp fraction of $f = 48 \pm 5\%$ contained in early endosomes.

From previous FRAP experiments the Dpp degradation rate was determined [2] to be $k_d = (2.52 \pm 1.29) \cdot 10^{-4}$ s$^{-1}$ [2]. The contribution of $k_{ex}$ was estimated from the Dpp production rate, which was found to be $2.69 \pm 1.58$ molecules per cell per second [2]. Since the system is in a steady state, the produced Dpp needs to be endocytosed at the same rate as it is produced and we set $k_{ex} = 2.69 \pm 1.58$ s$^{-1}$. This value is much faster than the typical endocytosis time, which typically is on the order of tens of seconds to a few minutes [26, 27]. The value reported here however is the average rate per Dpp molecule
if they would be endocytosed one by one. It therefore does not say anything about the amount of Dpp molecules that are endocytosed at the same time.

![Graph showing possible values for the recycling endosome outflow rate $k_r$ for different values of the early endosome outflow rate $k_{ea}$, using $f = 0.48$ and $\varepsilon = 0.996$ (solid line). The dotted lines show $k_r$ and $k_{ea}$ for values of $\varepsilon=0.994$ (1), $\varepsilon=0.995$ (2), $\varepsilon=0.997$ (3) and $\varepsilon=0.998$ (4). The dashed lines indicate the allowed values for $k_r$ and $k_{ea}$ if transport across a single cell takes 150 s as lower limit and 50 s as upper limit as estimated and measured before [2, 6, 28, 29]. The intersection of the solid and the dashed lines indicate range of solutions for the values of $1.0 \cdot 10^{-2} < k_r < 3.5 \cdot 10^{-2}$ s$^{-1}$ and $1.9 \cdot 10^{-2} < k_{ea} < 4.7 \cdot 10^{-2}$ s$^{-1}$.]

Using eq. (3.9) we further determined an upper limit value for the early endosomes outflow rate to be $k_{ea,max} = 0.13$ s$^{-1}$, which translates to a minimum Dpp residence time in early endosomes of $t_r = \frac{1}{k_{ea}} > 7.6$ s. Values for the early endosome outflow rate $k_{ea}$ and the recycling endosome outflow rate $k_r$...
are further limited by eq. (3.6). By realizing that both rates must be ultimately limited by the total rate estimated for Dpp transport across a single cell [6, 28, 29] which was $1/(50 \text{ s})$ and $1/(150 \text{ s})$, respectively, we find the constraint

$$50 < \frac{1}{k_{\text{ex}}} + \frac{1}{\varepsilon k_{\text{ea}}} + \frac{1}{k_{r}} < 150 \quad (3.14)$$

as indicated as dashed lines in fig. 3.6. We already determined that $k_{\text{ex}}$ is much faster compared to $k_{\text{ea}}$ and $k_{r}$. Hence, the contribution of $k_{\text{ex}}$ in eq. (3.14) is negligible. From the graph in fig. 3.6 we find that $1.9 \cdot 10^{-2} < k_{\text{ea}} < 4.7 \cdot 10^{-2} \text{ s}^{-1}$ and $1.0 \cdot 10^{-2} < k_{r} < 3.5 \cdot 10^{-2} \text{ s}^{-1}$. For the average residence times $t_{r}$ of Dpp in the early and recycling endosomes we found $21 < t_{r,\text{ea}} < 53 \text{ s}$ and $29 < t_{r,r} < 100 \text{ s}$, respectively. Uncertainty in $\varepsilon$, estimated to be 0.002, did not significantly influence this result (see dotted lines in fig. 3.6 for $\varepsilon = 0.994 (1)$, 0.995 (2), 0.997 (3), and 0.998 (4)).

### 3.6 Conclusion

![Diagram](image)

Figure 3.7: Summary of the obtained results.

Our data show that PICCS is a quantitative tool for addressing spatial and temporal correlations between interacting particles or proteins. The method alleviates restrictions which hampered previously developed methods. PICCS
reliably allowed us to measure the cross-correlation between two populations by which we arrived at quantitative insights into the subcellular mechanisms underlying intracellular Dpp transport. The results are summarized in fig. 3.7, where the estimations for the different rates are shown for the slow limit of intracellular transport. From the rates we found that the average residence time of Dpp in early endosomes is slightly shorter compared to recycling endosomes. Compared to the total life time of a Dpp molecule of 46 min [2] the average residence time in early and recycling endosomes is about 1 min for the slow limit and about 25 s for the fast limit. Furthermore we found that early endosomes contain on average almost twice as much Dpp compared to other endosomes. The results obtained are the first steps in a quantitative description of the transcytosis pathway in morphogen gradient formation.

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3.A Appendix

3.A.1 Theory - Intracellular trafficking

The steady-state concentration of Dpp in each type of endosome in given cell $n$ is calculated from eqs. (3.1) to (3.4)

$$C_{ea}^{n} = \frac{k_{ex} \left( C_{ex}^{n} + C_{ex}^{n+1} \right)}{2k_{ea}}$$  \hspace{1cm} (3.15a)

$$C_{r}^{n} = \frac{\varepsilon k_{ea} C_{ea}^{n}}{k_{r}}$$  \hspace{1cm} (3.15b)
Combining eqs. (3.15a), (3.15b) and (3.15d) gives

\[
C_{ex}^n = \frac{1}{4} \frac{\varepsilon}{1 - \varepsilon} \left( C_{ex}^{n-1} - 2C_{ex}^n + C_{ex}^{n+1} \right) \quad (3.16)
\]

If the Dpp gradient varies on length scales which are large compared to the size of a cell, we can approximately write the right side of the above equation as a second derivative, which results in

\[
C_{ex} = \frac{1}{4} \frac{\varepsilon}{1 - \varepsilon} \frac{d^2C_{ex}}{dn^2} \quad (3.17)
\]

with solution

\[
C_{ex}(n) = C_{ex}(0) e^{-\frac{n}{\lambda}} \quad (3.18)
\]

with the decay length \( \lambda \) in number of cells. Equation (3.16) and eq. (3.18) relate \( \varepsilon \) to \( \lambda \), with

\[
\varepsilon = \frac{2}{1 + \cosh (\lambda^{-1})} = 0.996 \quad (3.19)
\]

using the experimentally determined value for \( \lambda \) (\( \lambda = 7.7 \) cells, [2]).

The total concentration of Dpp in endosomes \( C_{tot,endo}^n \) in cell \( n \) is given by the sum of eqs. (3.15a) to (3.15c)

\[
C_{tot,endo}^n = \frac{k_{ex} k_r k_l + \varepsilon k_{ea} k_l k_{ex} + (1 - \varepsilon) k_{ea} k_{ex} k_r}{2k_{ea} k_r k_l} \times \left( 1 + e^{-\frac{n}{\lambda}} \right) C_{ex}^n = \text{const.} \times C_{ex}^n \quad (3.20)
\]

Thus the gradient of the intracellular Dpp concentration follows the extracellular gradient linearly.
3.A Appendix

3.A.2 Theory-Particle image cross-correlation spectroscopy

We consider 2 interaction partners whose fluorescence signals are labeled ‘YFP’ and ‘CFP’ without loss of generality. The goal is to find the correlation fraction and length from the spatial positions of the signals.

If $P(x, y)$ is the probability to find two correlated signals separated by a vector $(x, y)$, then the cumulative probability $P_{\text{cum}}(l)$ is found by integration of $P(x, y)$ in polar coordinates

$$P_{\text{cum}}(l) = 2\pi \int_0^l dr P(r) \quad (3.21)$$

with $r = \sqrt{x^2 + y^2}$.

The shape of the function $P_{\text{cum}}(l)$ depends on the nature of the interaction between the interaction partners and the positional accuracy for determination of the YFP and CFP signals. The experimentally observed $P(x, y)$ is found from the convolution of the real correlation $P_{\text{corr}}(x, y)$, which is characteristic for a specific interaction, and the probability density $P_{\text{pos. acc.}}(x, y)$ describing the (apparent) correlation due to the finite positional accuracy [30].

$$P(x, y) = \int \int dx' dy' P_{\text{corr}}(x - x', y - y') \times P_{\text{pos. acc.}}(x', y')$$

$$P_{\text{pos. acc.}}(x, y) = \frac{1}{2\pi\sigma^2} \exp\left( -\frac{x^2 + y^2}{2\sigma^2} \right) \quad (3.22)$$

where $\sigma = \sqrt{2}\delta$ and $\sigma\delta$ is the one-dimensional positional accuracy.

In the simplest case, if the YFP and CFP signal are at the same position, $P_{\text{cum}}(l)$ is determined by the positional accuracy alone:

$$P_{\text{cum}}(l) = 1 - \exp \left( -\frac{l^2}{2\sigma^2} \right) \quad (3.23)$$

A fit of Eq. 3.23 to $P_{\text{cum}}(l)$ with $\sigma$ as the free fit parameter results in a value for the one-dimensional positional accuracy $\sigma_{\text{pos. acc.}} = \sigma/\sqrt{2}$. More generally $\sigma$ can be regarded as a typical correlation length.
To accurately describe the experimentally determined $P_{\text{cum}}(l)$ we found that we had to assume two correlation lengths ($\sigma_1$ and $\sigma_2$)

$$P_{\text{cum}}(l) = \beta \left( 1 - \exp \left( -\frac{l^2}{2\sigma_1^2} \right) \right) + (1 - \beta) \left( 1 - \exp \left( -\frac{l^2}{2\sigma_2^2} \right) \right)$$

(3.24)

where $\beta$ is the fraction of data that has a correlation length $\sigma_1$. We suppose that a broad distribution of positional accuracies explains this functional form of $P_{\text{cum}}(l)$.

Now we derive step-by-step the form of the correlation function $C_{\text{cum}}(l)$ given above in Eq. 3.10. If, per image, there is only one pair of correlated signals (for clarity they will be called ‘YFP signal’ and ‘CFP signal’) the correlation function $C_{\text{cum}}(l)$ equals $P_{\text{cum}}(l)$: $C_{\text{cum}}(l) = P_{\text{cum}}(l)$. If only for a fraction $\alpha$ of all YFP signals there is a correlated CFP signal, we observe $C_{\text{cum}}(l) = \alpha P_{\text{cum}}(l)$. Typically there is more than one YFP signal per image and therefore also more than one CFP signal. If $l$ gets bigger, neighboring CFP signals in close proximity are counted by the PICCS algorithm although they are not correlated with the YFP signal. Additionally there might be CFP signals which are not correlated with any YFP signal. These CFP signals, in close proximity or not correlated with any YFP signal, lead to an additional contribution $c_{\text{CFP}} \cdot \pi l^2$ to $C_{\text{cum}}(l)$. Here we assume that the positions of the CFP signals follow a uniform random distribution with density $c_{\text{CFP}}$. In total $C_{\text{cum}}(l) = \alpha P_{\text{cum}}(l) + c_{\text{CFP}} \cdot \pi l^2$.

If there are no CFP signals in addition to the ones correlated with a YFP one, $c_{\text{CFP}}$ can be calculated from the density of YFP signals $c_{\text{YFP}}$, the correlation fraction $\alpha$ and the image area $A$ by

$$c_{\text{CFP}} = \alpha(c_{\text{YFP}}A - 1)/A = \alpha(c_{\text{YFP}} - 1/A) = c_{\text{CFP}}^*$$

(3.25)

If $\alpha/A \ll c_{\text{YFP}}$, $c_{\text{CFP}} \approx \alpha c_{\text{YFP}}$. In general $c_{\text{CFP}} = c_{\text{CFP}}^* + c_{\text{CFP, uncorr.}}$, where $c_{\text{CFP, uncorr.}}$ is the density of CFP signals which are not correlated with any YFP signal.
Non-random distribution of signals  In any real life situation, the assumption that all CFP signals are distributed randomly with a uniform density is often violated. One reason is the diffraction limit: if two molecules are too close to each other (< 200 nm) their fluorescence signals will merge and only one signal is observed. Consequently, close to a given CFP signal the probability to find another signal is decreased. Additionally, there might be biological reasons for correlations between the CFP signals. For example, receptors might be distributed evenly (and non-randomly) to achieve a very homogeneous surface coverage. Any correlation between the positions of CFP signals will cause a deviation from the simple quadratic contribution $c_{\text{CFP}} \cdot \pi l^2$ we assumed. The influence of this correlation on the cumulative correlation $C_{\text{cum}}(l)$ between the two color channels depends on the distribution of distances between YFP and CFP signals $P_{\text{cum}}(l)$. We define the function $s(r, l)$ as the number of CFP signals in a circle with radius $l$ if the distance between the YFP signal and a correlated CFP signal is $r$. For YFP signals which have a correlated CFP signal, the contribution of uncorrelated signals can be written as

$$\alpha \int_0^\infty dr \, s(r, l) \frac{\partial P_{\text{cum}}}{\partial r}(r)$$

(3.26)

where $\partial P_{\text{cum}}(r)/\partial r$ gives the probability for a distance $r$ between a pair of correlated signals. For YFP signals without correlated CFP signals, and assuming the simple quadratic dependence, we derive

$$C_{\text{cum}}(l) = \alpha P_{\text{cum}}(l) + (1 - \alpha) c \cdot \pi l^2$$

(3.27)

$$+ \alpha \int_0^\infty dr \, s(r, l) \frac{\partial P_{\text{cum}}}{\partial r}(r)$$

$s(r, l)$ is determined empirically from the experimental data by correlation of a 'virtual' YFP channel image with the measured images from the CFP channel. The virtual YFP image is constructed from the CFP image by placing YFP signals at a distance $r$ from a CFP signal. The $C_{\text{cum}}(l)$ determined for a given $r$ with the standard algorithm is equal to $s(r, l)$. Typically the results from 20 virtual images (where the YFP signals are moved around on circles with radius $r$ around the CFP signals) are averaged to obtain $s(r, l)$. 
Subsequent to the calculation of $s(r, l)$ the correction is determined numerically by the following self-consistent algorithm:

1. as an initial guess for the correction term determine the slope of the linear part of $C_{\text{cum}}$ and use the original correction term from eq. (3.10).

2. subtract the correction.

3. determine $\alpha$ as the average over the flat part of the resulting curve

4. normalize to 1 and fit the model eq. (3.11).

5. calculate the new correction according to eq. (3.27), go to step 2.

Steps 2 to 5 are repeated until the fit parameters change less than a predefined threshold.

### 3.A.3 Bleaching

Bleaching of the fluorescent label can influence the results. If one of the two fluorescent labels bleaches more quickly than the other, signals are lost and the correlation fraction will decrease over acquisition time. To confirm that the number of early endosomes (CFP signal) and Dpp-containing endosomes (YFP signal) stayed constant we measured the total number of detected endosomes per image stack (fig. 3.8). On average we detected 17 early endosomes in a field of view (1 early endosome per cell on average) and the number of observed endosomes was constant around this value. The average number of Dpp-containing endosomes found was 1.5 per cell. Since Dpp is also in the other endosomes we expected to find more Dpp-containing endosomes on average. Fluctuations in the number of detected endosomes were caused by movement of endosomes in and out of the image volume or by endosomes that were moving too close together to be detected individually. The latter effect is corrected for in the PICCS-algorithm.
Figure 3.8: Number of detected endosomes per image stack for a) early endosomes and b) Dpp-containing endosomes. The number of endosomes (signals) in both channels stayed approximately constant. The average number of endosomes and the standard deviation are indicated for both cases.

3.A.4 Error scaling in PICCS

To design a successful experiment it is crucial to know how the error of the measured observables (α, c_{CFP}, σ) scales with the experimental and fitting parameters (fig. 3.9). We determined the error by application of the PICCS algorithm described above to simulated data, assuming that the signals are distributed randomly and uniformly in space and the correlations are governed by eq. (3.11). First, we assume that all CFP signals are correlated with a YFP signal, so \( c_{CFP} = \alpha (c_{YFP} - 1/A) \), where \( A \) is the area of the image. Then we add additional CFP signals, which are not correlated with any YFP signal. For every set of parameters the simulations are repeated 100 times and the errors \( \Delta \alpha, \delta c_{CFP}, \Delta \sigma \) are determined as the standard deviation.

**Experimental parameters**   The experimental parameters are the correlation fraction \( \alpha \), the density of YFP and CFP signals \( c_{YFP} \) and \( c_{CFP} \), the correlation length \( \sigma \) and the number of images \( M \). As evident from fig. 3.10a all errors scale approximately like \( 1/\sqrt{M} \) where \( M \) is the number of acquired images. This behavior assures that any error can be made small just by acquisition of
more images. The same scaling behavior is found for $\alpha$, see fig. 3.10b. As to be expected, the relative errors become large if the correlation fraction is small or, equivalently, more images have to be acquired to achieve a certain accuracy. The dependence of the error on the density of YFP signals $c_{\text{YFP}}$ is different for the various observables (fig. 3.10c). While the error for $c_{\text{CFP}}$ scales like the inverse square root ($1/\sqrt{c_{\text{YFP}}}$), the errors of $\alpha$ and $\sigma$ are fitted with the empirical model $A \cdot \left(c_{\text{YFP}}/\mu\text{m}^{-2}\right)^{-0.5} + B \cdot \left(c_{\text{YFP}}/\mu\text{m}^{-2}\right)^{0.25}$. This model has a minimum at $(2A/B)^{4/3}$, which implies that there is an optimal density $c_{\text{YFP}}$, where the errors are minimal. As will become clear below, the value of this optimal density depends on the fitting parameters. The errors of $\alpha$ and $\sigma$ initially decrease with increasing $c_{\text{YFP}}$ because of the higher number of YFP signals, which increases statistical significance. At the same time, $c_{\text{CFP}}$ increases and therefore the con-
3.A Appendix

Distribution \( c_{\text{CFP}} \cdot \pi l^2 \) increases relative to \( \alpha \). Consequently, the errors of \( \alpha \) and \( \sigma \) increase for big densities \( c_{\text{YFP}} \).

So far, all CFP signals had a corresponding (i.e. correlated) YFP signal. Now we add additional, non-correlated CFP signals. If the density of YFP signals \( c_{\text{YFP}} \) and the interaction fraction \( \alpha \) are kept constant, \( \alpha \) and \( \sigma \) scale approximately like \( \sqrt{c_{\text{CFP}}} \) while the error of \( c_{\text{CFP}} \) scales like \( 1/\sqrt{c_{\text{CFP}}} \) (fig. 3.10d). As to be expected, the presence of extra CFP signals makes the determination of \( \alpha \) and \( \sigma \) increasingly difficult. A change in the correlation length \( \sigma \) has significant influence only on the error for \( \sigma \) which scales like \( 1/\sqrt{\sigma} \). For increasing \( \sigma \) there are more data points in a region which is important for the determination of \( \sigma \), namely where \( P_{\text{cum}}(l) \) is significantly smaller than 1. The errors of \( \alpha \) and \( c_{\text{CFP}} \) are approximately constant (fig. 3.10e).

**Fitting parameters** The fitting parameters are the length of the interval for the linear fit \( l_{\text{max}} - l_{\text{min}} \), its center \( l_{\text{center}} = (l_{\text{max}} - l_{\text{min}})/2 \) and the distance between two data points \( \Delta l \) (fig. 3.9). Figures 3.11a-c show that the general scaling behavior is independent on the position of the fit interval \( l_{\text{center}} \). However, the position of the minimum error of \( \alpha \) and \( \sigma \) depends on \( l_{\text{center}} \): The bigger \( l_{\text{center}} \), the smaller the optimal density \( c_{\text{YFP}} \). Therefore, \( l_{\text{center}} \) should be as small as the data allows - of course, the fit interval must be in the region where \( C_{\text{cum}}(l) \) is linear when plotted versus \( l^2 \). Figures 3.11d,e show the dependence on the errors on the length of the fit interval and the distance between data points respectively. The errors asymptotically become constant for big fit intervals and small distances between data points. Note that increasing \( l_{\text{max}} \) at constant \( l_{\text{min}} \) enlarges the fit interval but also moves its center \( l_{\text{center}} \), which is disadvantageous, see above.
Figure 3.10: a) Dependence of the relative errors on the number of images $M$. The relative errors of $\alpha$ (circles), $\sigma$ (triangles) and $c_{\text{CFP}}$ (squares) all scale approximately like $1/\sqrt{M}$ (solid line). $M = 10$, $\alpha = 0.5$, $c_{\text{YFP}} = 0.5 \, \mu m^{-2}$, $\sigma = 0.15 \, \mu m$ b) Dependence of the relative errors on the interaction fraction $\alpha$. The legend is the same as in fig. 3.10a, where $c_{\text{YFP}} = 1 \, \mu m^{-2}$ (closed symbols), $c_{\text{YFP}} = 10 \, \mu m^{-2}$ (open symbols), $M = 10$, $\sigma = 0.15 \, \mu m$ in both cases. The errors of all determined parameters approximately scale like $1/\sqrt{\alpha}$ (solid line), independent of the density $c_{\text{YFP}}$. c) Dependence of the relative errors on the density $c_{\text{YFP}}$. The legend is the same as in fig. 3.10a. The relative error of $\alpha$ (circles) and $\sigma$ (triangles) are fitted with the model $A \cdot (c_{\text{YFP}}/\mu m^{-2})^{-0.5} + B \cdot (c_{\text{YFP}}/\mu m^{-2})^{0.25}$ (black solid and dashed line respectively). For $\alpha A = 0.04$, $B = 0.12$, which results in a minimum at 0.6 $\mu m^{-2}$ and for $\sigma A = 0.07$, $B = 0.14$, which gives a minimum at 0.5 $\mu m^{-2}$. The relative error of $c_{\text{CFP}}$ (squares) scales approximately like $c_{\text{YFP}}^{-2/3}$ (The gray solid line is a linear fit in the logarithmic plot given by $y = -0.66(c_{\text{YFP}}/\mu m^{-2}) - 2.9$). $M = 10$, $\alpha = 0.5$, $\sigma = 0.15 \, \mu m$. d) Dependence of the relative errors on the density $c_{\text{CFP}}$. The legend is the same as in fig. 3.10a. The relative error of $\alpha$ and $\sigma$ scale approximately like $\sqrt{c_{\text{CFP}}}$ (solid line), the relative error of $c_{\text{CFP}}$ scales like $1/\sqrt{c_{\text{CFP}}}$ (dashed line). $M = 10$, $c_{\text{YFP}} = 1 \, \mu m^{-2}$, $\alpha = 0.5$, $\sigma = 0.15 \, \mu m$. e) Dependence of the relative errors on $\sigma$. The legend is the same as in fig. 3.10a. The relative error of $\alpha$ and $c_{\text{CFP}}$ do not change significantly with $\sigma$. The relative error of $\sigma$ scales approximately like $1/\sqrt{\sigma}$ (The solid line is a linear fit in the logarithmic plot given by $y = -0.52(c_{\text{YFP}}/\mu m^{-2}) - 2.8$). $M = 50$, $c_{\text{YFP}} = 1 \, \mu m^{-2}$, $\alpha = 0.5$. 
Figure 3.11: a) Dependence of the relative error of $\alpha$ on the center of the fit interval $l_{\text{center}} = (l_{\text{max}} - l_{\text{min}})/2$. The legend is the same as in fig. 3.10a, where $l_{\text{center}} = 0.925$ (solid symbols), $l_{\text{center}} = 1.175$ (gray symbols) and $l_{\text{center}} = 1.375$ (open symbols). The relative error of $\alpha$ is fitted with the model $A \cdot (c_{\text{YFP}}/\mu \text{m}^{-2})^{-0.5} + B \cdot (c_{\text{YFP}}/\mu \text{m}^{-2})^{0.25}$. $A = 0.04$, $B = 0.05$ (black solid line), $A = 0.04$, $B = 0.09$ (gray solid line) and $A = 0.04$, $B = 0.13$ (dashed line). That results in minima at 1.9 $\mu \text{m}^{-2}$, 0.9 $\mu \text{m}^{-2}$ and 0.5 $\mu \text{m}^{-2}$ respectively. $M = 10$, $\alpha = 0.5$, $\sigma = 0.05 \mu \text{m}$. b) Dependence of the relative error of $\sigma$ on the center of the fit interval $l_{\text{center}} = (l_{\text{max}} - l_{\text{min}})/2$. The legend is the same as in fig. 3.10a, where $l_{\text{center}} = 0.925$ (solid symbols), $l_{\text{center}} = 1.175$ (gray symbols) and $l_{\text{center}} = 1.375$ (open symbols). The relative error of $\sigma$ is fitted with the model $A \cdot (c_{\text{YFP}}/\mu \text{m}^{-2})^{-0.5} + B \cdot (c_{\text{YFP}}/\mu \text{m}^{-2})^{0.25}$. $A = 0.07$, $B = 0.15$ (black solid line), $A = 0.08$, $B = 0.25$ (gray solid line) and $A = 0.09$, $B = 0.34$ (dashed line). That results in minima at 0.9 $\mu \text{m}^{-2}$, 0.6 $\mu \text{m}^{-2}$ and 0.4 $\mu \text{m}^{-2}$ respectively. $M = 10$, $\alpha = 0.5$, $\sigma = 0.05 \mu \text{m}$. c) Dependence of the relative error of $c_{\text{CFP}}$ on the center of the fit interval $l_{\text{center}} = (l_{\text{max}} - l_{\text{min}})/2$. The legend is the same as in fig. 3.10a, where $l_{\text{center}} = 0.925$ (solid symbols), $l_{\text{center}} = 1.175$ (gray symbols) and $l_{\text{center}} = 1.375$ (open symbols). The relative error of $\sigma$ is fitted with the straight line (in the logarithmic plot). The slope is $-0.77$ (black solid line) $-0.67$ (gray solid line) and $-0.62$ (dashed line). $M = 10$, $\alpha = 0.5$, $\sigma = 0.05 \mu \text{m}$. d) Dependence of the relative errors on the length of the fit interval $(l_{\text{max}} - l_{\text{min}})$. The legend is the same as in fig. 3.10a. $M = 50$, $\alpha = 0.5$, $\sigma = 0.15 \mu \text{m}$, $c_{\text{YFP}} = 1 \mu \text{m}^{-2}$. e) Dependence of the relative errors on the step size $\Delta l$ (fig. 3.9). The legend is the same as in fig. 3.10a. $M = 50$, $\alpha = 0.5$, $\sigma = 0.15 \mu \text{m}$, $c = 1 \mu \text{m}^{-2}$.
Bibliography


