Appendix: Instrumental noise in the analysis of granule motion

The measurements of integrated intensity fluctuations observed frame-to-frame from individual granules are affected not only by the actual motion of granules but also by instrumental noise, mainly photon shot noise and CCD camera readout noise and dark count noise. The instrumental noise has two effects on the estimation of $<(\Delta z)^2>$ or $<(\Delta R)^2>$, which are measures of the amount of z-motion or R-motion in the time duration of a camera frame interval. The first effect is an overestimate of $<(\Delta z)^2>$ or $<(\Delta R)^2>$, because some part of the fluctuations arise purely from instrumental noise: even an absolutely fixed granule would appear to be moving. This overestimation bias can be corrected as described below. The second effect is an increase in the statistical uncertainty of results for $<(\Delta z)^2>$ or $<(\Delta R)^2>$, even after correction for the overestimation bias. This effect is also evaluated below. Any quantitative conclusions based on noise analysis of intensity data must take these two effects into account, in order to correctly report unbiased results and also to correctly evaluate whether values for motions under different biological conditions are significantly different from each other or from zero.

The correction and assignment of uncertainties for $<(\Delta z)^2>$ and for $<(\Delta R)^2>$ are somewhat different from each other so they are described separately.

Overestimation correction for $<(\Delta z)^2>$

The primed variables here refer to the observables (i.e., before correction for instrumental noise) and the unprimed variables refer to actual motion. The intensities in successive frames I_1 and I_2 are already assumed to be background-subtracted.

At any time i,

$$I_i' = I_0 e^{-z_i'/d} \tag{A1}$$

where z_i' is the *apparent* z-position. In terms of the *actual* z-position, we have:

$$I_i' = \alpha I_0 e^{-z/d} \tag{A2}$$

where α is a random variable representing instrumental noise that fluctuates around $\langle\alpha\rangle=1$ at any particular intensity. The statistical behavior of random variable α in general is a function of mean intensity; its value and statistical behavior is essentially the same as the normalized intensities seen in the experiments on immobilized debris or beads, samples which have no actual motion. From Eq. A1 and A2 evaluated at the times i=1,2 of two successive camera exposures and taking the ratio, we get

$$e^{-\Delta z'/d} = \frac{\alpha_2}{\alpha_1} e^{-\Delta z/d} \tag{A3}$$

where $\Delta z \equiv z_2 - z_1$ and likewise for $\Delta z'$. Taking the log of Eq. A3 gives

$$\frac{\Delta z'}{d} = \frac{\Delta z}{d} + \ln\alpha_2 - \ln\alpha_1 \tag{A4}$$

The three terms on the right are all random variables that are completely independent of each other. In such a situation, the variance of a sum (or difference) is the sum of the variances. Therefore, their variances all add to produce the variance of the quantity on the left:

$$\operatorname{var}\frac{\Delta z'}{d} = \operatorname{var}\frac{\Delta z}{d} + 2\operatorname{var}\ln\alpha \tag{A5}$$

Note that in general $\operatorname{var}\Delta z = \left\langle \Delta z^2 \right\rangle - \left\langle \Delta z \right\rangle^2$, and likewise for $\operatorname{var}\Delta z'$, so

$$\left\langle \left(\frac{\Delta z'}{d}\right)^2 \right\rangle = \left\langle \left(\frac{\Delta z}{d}\right)^2 \right\rangle + 2\operatorname{var}\left(\ln\alpha\right) + \left\langle \frac{\Delta z'}{d} \right\rangle^2 - \left\langle \frac{\Delta z}{d} \right\rangle^2 \tag{A6}$$

If any unidirectional motion (say, favoring motion toward the membrane) underlies the random motions, the last two terms on the right each may be nonzero. However, the difference between them is very small, as can be shown by taking the mean values on each side of Eq. A4 and examining $\langle \ln \alpha_2 \rangle - \langle \ln \alpha_1 \rangle$. The extreme maximum value of $\langle \ln \alpha_2 \rangle - \langle \ln \alpha_1 \rangle$ for any two intensity ranges, as calculated from noise read on immobilized beads of varying intensities with our CCD detector system, is less than 0.06. This means that the misreporting of any mean unidirectional motion $\langle \Delta z \rangle$ due to CCD instrumental noise will be less than 6% of the characteristic depth *d*. Consequently, the last two terms on the right side of Eq. A6 almost cancel each other and can be safely ignored, even in the presence of unidirectional motion.

Eq. A6 can be further simplified in the case where the fluctuations in α around its mean value of unity are small, as can be checked from the relative size of frame-to-frame intensity fluctuations on immobile debris or beads. In that case, $\ln \alpha \approx \alpha - 1$, and the last term in Eq. A6 becomes var α, giving the approximate result:

$$\left\langle (\Delta z')^2 \right\rangle = \left\langle (\Delta z)^2 \right\rangle + 2d^2 \operatorname{var}(\alpha) \qquad \text{(A7)}$$

Therefore, to calculate a particular granule's corrected $\langle(\Delta z)^2\rangle$ from the uncorrected $\langle(\Delta z')^2\rangle$, we measure a sequence of intensities on a debris/bead sample, normalize the sequence to the average intensity, calculate the variance, multiply by $2d^2$, and subtract this value from the uncorrected $\langle(\Delta z')^2\rangle$. Note that this procedure must be done with α values measured on a debris/bead sample that has the same mean intensity as the granule.

Overestimation correction for $\langle(\Delta R)^2\rangle$

The values for $\Delta R'$ are determined by quadrature from the component measurements of $\Delta X'$ and $\Delta Y'$. Each of those readings contains a "real" motion ($\Delta X, \Delta Y$) denoted as unprimed, and a 'noise' motion ($\Delta\beta_x$, $\Delta\beta_y$). The noise motion is the motion inferred from measurements on immobilized beads of similar size and intensity to a granule.

$$\begin{aligned} \Delta X' &= \Delta X + \Delta\beta_x \\ \Delta Y' &= \Delta Y + \Delta\beta_y \end{aligned} \tag{A8}$$

The frame-to-frame fluctuations in ΔX and ΔY are presumed to be uncorrelated with each other, and also uncorrelated with the two uncorrelated instrumental noise fluctuations $\Delta\beta_x$ and $\Delta\beta_y$. Therefore,

$$\begin{aligned} \operatorname{var}\Delta X' &= \operatorname{var}\Delta X + \operatorname{var}\Delta\beta_x \\ \operatorname{var}\Delta Y' &= \operatorname{var}\Delta Y + \operatorname{var}\Delta\beta_y \end{aligned} \tag{A9}$$

Since the means of each of the random variable distances are zero, we have

$$\begin{aligned} \left\langle (\Delta X')^2 \right\rangle &= \left\langle (\Delta X)^2 \right\rangle + \left\langle (\Delta\beta_x)^2 \right\rangle \\ \left\langle (\Delta Y')^2 \right\rangle &= \left\langle (\Delta Y)^2 \right\rangle + \left\langle (\Delta\beta_y)^2 \right\rangle \end{aligned} \tag{A10}$$

The sum of these two equations is $<(\Delta R')^2>$, giving

$$\left\langle (\Delta R')^2 \right\rangle = \left\langle (\Delta R)^2 \right\rangle + \left\langle (\Delta\beta)^2 \right\rangle \tag{A11}$$

Therefore, to calculate a particular granule's corrected $<(\Delta R)^2>$, we subtract the average radial motion $<(\Delta\beta)^2>$ measured on a bead of similar size and intensity from $<(\Delta R')^2>$ measured on a granule.

Statistical accuracy of corrected $<(\Delta z)^2>$

After correction for instrumental noise as above, the now-unbiased estimates for $\langle(\Delta z)^2\rangle$ vs. z (or intensity) still contain an uncertainty which arises from the random nature of z compounded by random instrumental noise. We estimate this uncertainty by a simulation program with random number generation.

The first step is to simulate the instrumental noise in intensity as a combination of photon count-independent CCD camera readout noise and photon count-dependent shot noise. The parameters describing this combination are phenomenologically set to produce photon count histograms that agree with the series of histograms observed experimentally on debris/bead samples.

Next, actual "granule" motions are simulated by generating a series of Δz values where the z-motion steps are assumed to be Gaussian-distributed variables with variances corresponding to the corrected values found in the experiments at each particular mean intensity (or z) range. These simulated positions are converted to intensity, at which point simulated readout and shot noise are folded in according to the phenomenological parameters determined as in the above paragraph, and then converted back to $\Delta z'$, the set of which is now even noisier than the original Δz. The number of motions simulated at each mean intensity is set to be the same as the number that were actually accumulated at that intensity range in the experiments on chromaffin cell granules. With this set of simulated $\Delta z'$ values, the corresponding simulated $\langle(\Delta z')^2\rangle$ could be calculated. Then the simulation is repeated again and again, each time giving new $\langle(\Delta z')^2\rangle$ values (because the average is over a finite number of random values). The variance of this set, var $\langle(\Delta z')^2\rangle$, could then be evaluated. The square root of this variance is the standard error (SE) in $\langle(\Delta z)^2\rangle$. Twice that SE represents the 95% confidence range shown in Fig. 1.

An alternative method that does not use simulation is based entirely on the variability in set of experimental $\Delta z'$ values at each intensity, using the definition of variance of the mean:

$$\mathrm{var}\left\langle \left(\Delta z'\right)^2 \right\rangle = \frac{\left\langle \left[\left(\Delta z'\right)^2 - \left\langle \left(\Delta z'\right)^2 \right\rangle \right]^2 \right\rangle}{n-1} \tag{A11}$$

where n is the number of experimental $\Delta z'$ values that fall into a particular range of intensities. This method is only useful if there are enough such values in the intensity range to produce reliable statistics.

Statistical accuracy of corrected $\langle(\Delta R)^2\rangle$

Actual granule motion steps in the x and y directions are each simulated as a Gaussian distributed variable with a variance corresponding to the corrected values found in the experiments at each particular intensity (or z) range. To this is added a random Gaussian-distributed step with a variance corresponding to the apparent "motion" of an immobile experimental bead arising from instrumental noise. From this sum, a $\Delta R'$ step is generated. The number of $\Delta R'$ motions simulated at each mean intensity is set to be the same as the number that were actually accumulated at that intensity range in the experiments on chromaffin cell granules. From this point on, the protocol for estimating the SE of the simulated values for $\langle(\Delta R)^2\rangle$ is analogous to that for $\langle(\Delta z)^2\rangle$ described above. The alternative method, based on actual variability in the set of experimental $\Delta R'$ values and an analogy to Eq. A11, can also be used.

Shot noise artifact in the estimation of sequential motions

To determine whether the motion of a granule is *directed* rather than completely random, the interframe granule motion measured between two successive frames at times t and t+1 ideally should be correlated with the immediately successive interframe motion measured between frames $t+1$ and $t+2$. In principle, a positive correlation shows that the motion persists in the same direction; a negative correlation shows that the motion tends to reverse itself; and a zero correlation indicates a random walk. In practice, however, a complication arises due to shot noise, which adds a randomness to the position measurement at any particular time, even if the granule is not moving at all. This complication gives rise to an apparent negative correlation in inferred granule motion between successive interframe intervals, even where one does not really exist. This effect occurs for both Δz and ΔR motions; the discussion here will refer to Δz for concreteness but it could be replaced by ΔR throughout.

First, assume shot noise is the only source of fluctuations in z. *Given* a shot noise generated "up-fluctuation" in the estimation for z at time $t+1$, the measured Δz_1 [$\equiv z(t+1)-z(t)$] in the $(t,t+1)$ interval will tend to be *positive* because the immediately prior $z(t)$ measurement is equally likely to be an "up" or a "down" fluctuation. The immediately subsequent measured Δz_2 [$\equiv z(t+2)-z(t+1)$] then will tend to be *negative* because $z(t+2)$ is equally likely to be an "up" or a "down" fluctuation. An analogous argument applies given a shot noise generated "down-fluctuation" in $z(t+1)$, with the "positive" and "negative" words reversed. Therefore, Δz_1 and Δz_2 are negatively correlated. Qualitatively, the negative correlation arises because calculation of Δz_1 and Δz_2 both share the same input measurement $z(t+1)$, one with a plus sign and the other with a minus sign. This artifact does *not* occur in comparing Δz_3 [$\equiv z(t+3)-z(t+2)$] with Δz_1, nor in comparing Δz_4 [$\equiv z(t+4)-z(t+3)$] with Δz_2.

The same conclusions apply even if the shot noise is mixed with actual motions in z. We assume that the measured $\Delta z'$ is composed of two independent parts: an actual z motion Δz and a shot noise contribution Δs:

$$\Delta z' = \Delta z + \Delta s \tag{A12}$$

The temporal autocorrelation function $G_{\Delta z'}$ for $\Delta z'$ is:

$$\begin{aligned} G_{\Delta z'}(\tau) &= \langle \Delta z'(t+\tau)\Delta z'(t) \rangle \\ &= \langle [\Delta z(t+\tau) + \Delta s(t+\tau)][\Delta z(t) + \Delta s(t)] \rangle \\ &= G_{\Delta z}(\tau) + G_{\Delta s}(\tau) \end{aligned} \tag{A13}$$

The cross terms in Eq A13 are zero since the actual motions and shot noise are uncorrelated. Variable s is the deviation from the actual z at any particular frame due to shot noise, and can be positive or negative. The autocorrelation of the interframe shot noise is:

$$\begin{aligned} G_{\Delta s} &= \langle \Delta s(t+\tau)\Delta s(t) \rangle \\ &= \langle [s(t+\tau+1) - s(t+\tau)][s(t+1) - s(t)] \rangle \\ &= \langle s(t+\tau+1)s(t+1) - s(t+\tau)s(t+1) - s(t+\tau+1)s(t) + s(t+\tau)s(t) \rangle \\ &= G_s(\tau) - G_s(\tau-1) - G_s(\tau+1) + G_s(\tau) \\ &= 2G_s(\tau) - G_s(\tau-1) - G_s(\tau+1) \end{aligned}$$

(A14)

$G_s(\tau)$ has the well-known form of a positive delta function spike at $\tau = 0$; shot noise exhibits no correlation with itself from one instant to the next. The second term and the third term in Eq. A14 produce negative spikes at $\tau = 1$ and $\tau = -1$, respectively. It is these terms that represent the artifactual negative correlation at $\tau = \pm 1$ in $G_{\Delta z'}(\tau)$ in Eq. A13. There is no artifact at any other nonzero τ value.

Johns et al (2001) show $G_{\Delta z'}(\tau)$ curves for z motion of secretory granules that display the negative correlation spike at $\tau = 1$. These were misinterpreted as being representative of actual z motions, but they are in fact shot noise artifacts. However, whether the artifact actually appears depends on whether shot noise in intensity measurements is significant compared to the "noise" in intensity measurements generated by actual z motions. In the case of very bright objects, where the relative size of shot noise is small, the negative artifact at $\tau = 1$ may not be noticeable. In principle, the problem can be surmounted by calculating an autocorrelation of a modified $\Delta_k z'$ ([$\equiv z'(t+k)-z(t)$] so that the negative spike artifact occurs at $\tau = \pm k$, where k represents a jump of many frames rather than just a single frame. If k is large enough, the artifact could be positioned beyond the temporal zone where actual z motions are correlated, and thereby its contribution could be quantified.