General Principles of Two Dimensional (2D) NMR

In NMR the most useful information comes from the interactions between two nuclei, either through the bonds which connect them (J-coupling interaction) or directly through space (NOE interaction). One can look at these interactions one at a time by irradiating one resonance in the proton spectrum (either during the relaxation delay or during acquisition) and looking at the effect on the intensity or coupling pattern of another resonance. 2D NMR essentially allows us to irradiate all of the chemical shifts in one experiment and gives us a matrix or two-dimensional map of all of the affected nuclei. There are four steps to any 2D experiment:

1. **Preparation**: Excite nucleus **A**, creating magnetization in the x-y plane
2. **Evolution**: Measure the chemical shift of nucleus **A**.
3. **Mixing**: Transfer magnetization from nucleus **A** to nucleus **B** (via J or NOE).
4. **Detection**: Measure the chemical shift of nucleus **B**.

Of course, all possible pairs of nuclei in the sample go through this process at the same time. **Preparation** is usually just a 90^o pulse which excites all of the sample nuclei simultaneously. **Detection** is simply recording an FID and finding the frequency of nucleus **B** by Fourier transformation. To get a second dimension, we have to measure the chemical shift of nucleus **A** *before* it passes its magnetization to nucleus **B**. This is accomplished by simply waiting a period of time (called t_1, the **evolution** period) and letting the nucleus **A** magnetization rotate in the x-y plane. The experiment is repeated many times over (for example, 512 times), recording the FID each time with the delay time t_1 incremented by a fixed amount. The time course of the nucleus **A** magnetization as a function of t_1 (determined by its effect on the final FID) is used to define how fast it rotates and thus its chemical shift. **Mixing** is a combination of RF pulses and/or delay periods which induce the magnetization to jump from **A** to **B** as a result of either a J coupling or an NOE interaction (close proximity in space). Different 2D experiments (e.g., NOESY, COSY, HETCOR, etc.) differ primarily in the mixing sequence, since in each one we are trying to define the relationship between **A** and **B** within the molecule in a different way.

The raw data from a 2D experiment consist of a series of FIDs, each acquired with a slightly longer t_1 delay than the previous one. Varian creates an array of FIDs, with the t_1 delay (parameter **d2**) arrayed. The first step in processing a 2D dataset is to Fourier transform each of the FIDs in the array. The resulting spectra are loaded into a matrix with the rows representing individual spectra in order of t_1 value, with the smallest t_1 value as the bottom row (Figure 1, next page). The horizontal axis is labelled F_2, which is the chemical shift observed directly in each FID, and the vertical axis is t_1, the evolution delay. Each row in the 2D matrix represents a spectrum acquired with a different t_1 delay, and each column in the matrix represents either noise (if the F_2 value of that column is in a noise region of the spectrum) or, if $F_2 = F_B$, the column is a t_1 "FID" with maximum intensity at the bottom and oscillating in a decaying fashion as we move up to higher t_1 values (Figure 1). The frequency of this oscillation is just the chemical shift of nucleus **A**. Of course, a real sample has more than one peak in its spectrum, so there would be other columns containing different t_1 FIDs.

The second step in processing the 2D data is to perform a second Fourier transform on each of the columns of the matrix. Most of columns will represent noise, but when we reach a column which falls on an F_2 peak, transformation of the t_1 FID gives a spectrum in F_1, with a peak

at the chemical shift of nucleus **A** (Figure 2). The final 2D spectrum is a matrix of numerical values which has a pocket of intensity at the intersection of the horizontal line $F_1 = F_A$ and the vertical line $F_2 = F_B$ and has an overall intensity determined by the efficiency of transfer of magnetization from nucleus **A** to nucleus **B**. This efficiency tells us something about the relationship (J value or NOE intensity) between the two nuclei within the molecule. Simultaneously with the process we described, other pairs of nuclei are undergoing the same evolution, mixing and detection process resulting in other crosspeaks at the intersections of the appropriate chemical shift lines and with characteristic intensities representing the efficiency of transfer of magnetization. The 2D spectrum thus represents a complete map of all interactions which lead to magnetization transfer, with the participants in the interaction addressed by their chemical shifts.

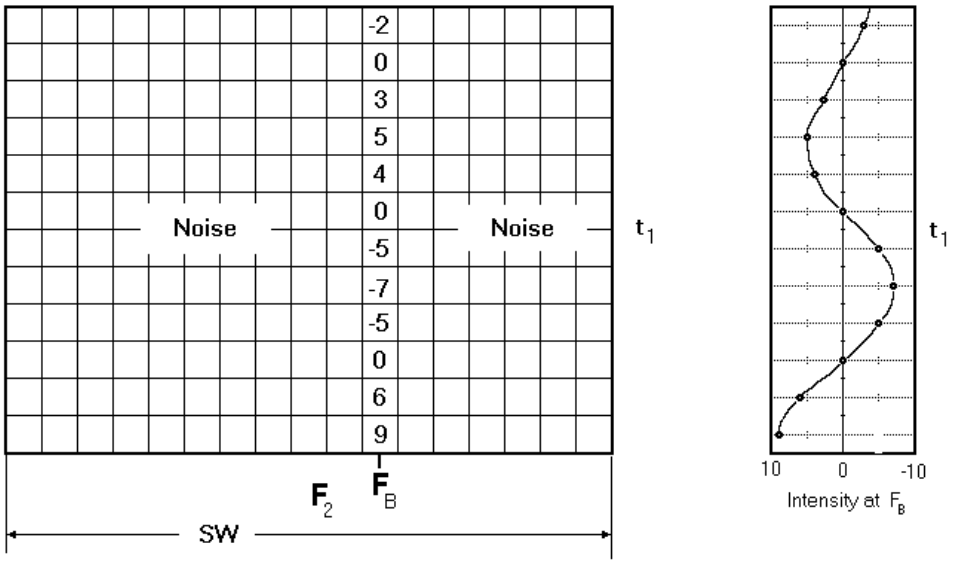

Figure 1. 2D Matrix after Fourier transform in F_2. The numbers represent data values at various cells in the data matrix, with the blank cells having zero or very small values.

All of the 2D experiments in use can be classified by the two types of nuclei detected in the direct (F_2) and indirect (F_1) dimensions and the criteria for magnetization transfer during the mixing step. The mixing pulse sequence is designed to select for certain types of interactions between nuclei, and can be divided into two categories: magnetization transfer based on a J-coupling interaction, and magnetization transfer based on an NOE interaction.

Name	F_1 nucleus	Mixing	F_2 nucleus
COSY	^{1}H	J	^{1}H
RELAY	^{1}H	J, J	^{1}H
TOCSY	^{1}H	J, J, J...	^{1}H
NOESY	^{1}H	NOE	^{1}H
HETCOR	^{1}H	$^{1}J_{CH}$	^{13}C
HMQC	^{13}C	$^{1}J_{CH}$	^{1}H
HMBC	^{13}C	$^{2,3}J_{CH}$	^{1}H

Experiments which transfer magnetization from one nucleus to another nucleus of the same type, e.g., ^1H to ^1H, are called homonuclear experiments, and are characterized by a **diagonal** defined by $F_1 = F_2$ and by pairs of crosspeaks at symmetrical positions across the diagonal: $F_1=F_A$, $F_2=F_B$ and $F_1=F_B$, $F_2=F_A$. This is because both magnetization transfer pathways, ^1H_A --> ^1H_B and ^1H_B --> ^1H_A, can be observed. Experiments which transfer magnetization

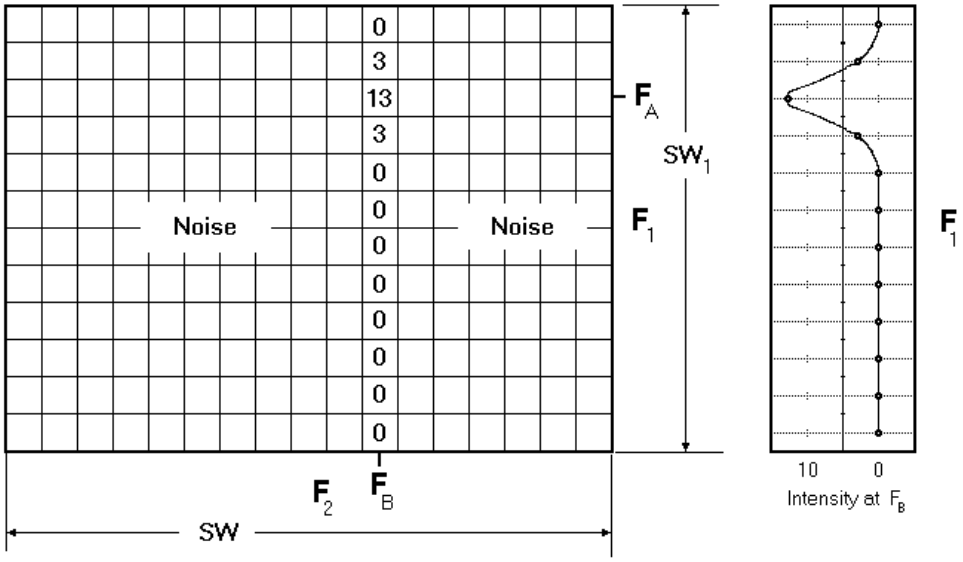

Figure 2. 2D matrix after Fourier transform in F_1. The crosspeak at $F_2 = F_B$, $F_1 = F_A$ is interpreted as an interaction between nucleus **A** and **B** which led to transfer of magnetization from nucleus **A** to nucleus **B** during the mixing period.

between two different types of nucleus, e.g., ^1H and ^{13}C, are called heteronuclear and lack a diagonal and diagonal symmetry. The range of J values can be selected in mixing schemes: for ^1H-^{13}C couplings, $^1J_{CH}$ (the one-bond or direct coupling) is very large (125-160 Hz) while the long-range $^{2,3}J_{CH}$ (two and three-bond coupling) is small (2-12 Hz). Mixing sequences can allow multiple "jumps" of magnetization: RELAY is set up for two jumps based on J coupling - one from the F_1 nucleus to an intermediated (undetected) nucleus and a second jump to the F_2 nucleus. The TOCSY experiment allows for many jumps, thus spreading the magnetization out over an entire "spin system" or group of protons interconnected by J couplings. From these basic experiments have grown many variants. The COSY has been extended to DQF-COSY (reduced diagonal and improved phase properties) and COSY-35 (simplified crosspeak structure for J-value determination). A common variant of NOESY is the ROESY, which gives better results for molecules in the size range of peptides, oligosaccharides and large natural products. HSQC gives the same results as HMQC but has better relaxation properties for large molecules such as proteins. All 2D experiments and even 3D and higher-dimensional experiments are based on the above list of basic 2D experiments.

In all this complexity of acronyms it is easy to forget that all 2D experiments do the same thing: they allow you to correlate two atoms (nuclei) in a molecule based on an interaction which is either through-bond (J-coupling) or through-space (NOE). The two nuclei are identified by their chemical shifts, and the correlation appears in the 2D spectrum as a crosspeak at the F_1

chemical shift ("y coordinate") of the nucleus where magnetization starts and the F_2 chemical shift ("x coordinate") of the nucleus to which the magnetization is transferred. Thus the basis of all 2D experiments is the "jump" or transfer of magnetization. The information (J value or NOE intensity) can be used to define structural relationships (dihedral angle or distance) but is only useful if we can unambiguously assign the two chemical shifts (F_1 and F_2) to specific positions within the molecule.

Bruker DRX Parameters for 2D NMR. Bruker generally uses the same parameter names (**sw**, **td**, **si**, **wdw, ssb**, etc.) for both (F_2 and F_1) dimensions of a 2D experiment. In the acquisition parameter menu (**eda**) and the processing parameter window (**edp**) there are two columns of parameters, the left-hand column for F_2 parameters and the right-hand column for F_1 parameters. You can also specify parameters at the command line by entering the parameter name (for the F_2 dimension) or a 1 followed by the parameter name (for the F_1 dimension). For example:

sw 7 <return>
1 sw 150 <return>

would set the spectral width to 7 ppm in the F_2 dimension and 150 ppm in the F_1 dimension. The offset parameters which set the center of the spectral window are exceptions to this rule – they are named according to the RF channel used in each dimension. For example, the HSQC experiment is a heteronuclear 2D experiment: the observe (F_2) dimension is ^{1}H and the indirect (F_1) dimension is ^{13}C. Bruker designates these two RF channels as f1 (^{1}H) and f2 (^{13}C). This refers to RF frequency 1 and RF frequency 2, not to be confused with the chemical shift scales F_2 and F_1 in the 2D spectrum. For this example the center of the spectral window in the F_2 (^{1}H) dimension is set by the parameter **o1** (offset for f1 channel in Hz) or **o1p** (in ppm). The center of the spectral window in the F_1 (^{13}C) dimension is set by the parameter **o2** (offset for f2 channel in Hz) or **o2p** (in ppm). This is pretty confusing but at least it's consistent: the f1 (first RF channel) is always the observe (F_2) dimension. In the case of homonuclear 2D experiments (e.g., DQF-COSY), there is only one RF channel: the proton (observe = f1) channel. In this case **o1** (in Hz) and **o1p** (in ppm) determine the center of the spectral window in both the F_2 and the F_1 dimensions.

To understand 2D data processing, you will have to review the techniques of **window (multiplier) functions** and **zero-filling**. These are 1D data processing techniques but they are much more commonly used in 2D processing. Because of the large file sizes associated with 2D experiments, it is sometimes necessary to use a small number of data points (**td**) in the acquired FID. This can lead to the phenomenon of *truncation*, which means that the FID is cut off before the signal has decayed to zero. Truncation is even more common in the t_1 (indirect) dimension, because each data point requires a complete acquisition of **ns** transients and time limitations usually make it impossible to acquire the full t_1 FID. One consequence of truncation is that the transformed spectrum has too few data points: peaks and multiplets are poorly defined because the digital resolution (number of data points per Hertz) is so low that only one or two data points are available to describe the shape of a peak in the spectrum. The digital resolution of the spectrum can be improved by a process called **zero-filling**. The FID is extended from **td**/2 data pairs (complex pairs) to **si** ("size") data points by simply adding zeroes as data values for the new

data points. Fourier transformation of the new, larger FID dataset gives a spectrum with many more points spread out evenly over the spectral width (Fig. 3). Note than the value of **si** is limited to powers of 2 (e.g., 512, 1024, 2048, 4096, etc.).

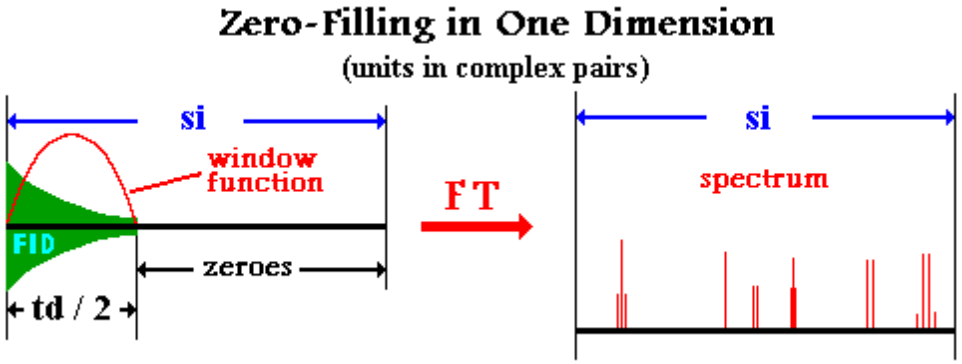

Figure 3. The truncated FID is extended in time by adding new data points with zero intensity values. Fourier transformation yields a spectrum with the same number of data points as the extended FID. In order to avoid "wiggles" around each peak in the spectrum which result from the abrupt truncation of the FID, a window (multiplier) function is used to force the FID data to come smoothly to zero at the end of the acquired data.

One problem remains when a truncated FID is zero-filled and Fourier-transformed. The abrupt drop at the end of the acquired data (**td** points) to the zero level of the added data points is converted by Fourier transformation into a series of wiggles around the peak in the spectrum. These "truncation wiggles" can extend outward and interfere with other peaks in the spectrum. To eliminate the truncation wiggles, a window or multiplier function is applied to the acquired FID data to force the FID to come smoothly to zero at the end of **td** data points. Two commonlyused window functions which accomplish this are the sine-bell and the cosine-bell (or 90^{o} shifted sine-bell) functions (Fig. 4). The cosine-bell function starts at the maximum (sine of 90^{o}) at the beginning of the FID and goes smoothly to zero (sine of 180^{o}) at the end of the acquired data. This window function is commonly used for HETCOR, NOESY, and TOCSY data. The sine-bell function starts at the zero point of the sine function (sine of 0^{o}) at the start of the FID data, reaches a maximum halfway through the FID (sine of 90^{o}) and falls back smoothly to zero by the end of the acquired FID data (sine of 180^{o}). Because data later in the FID (in the center) is emphasized over data early on in the FID, this window function leads to **resolution enhancement** at the expense of sensitivity (signal-to-noise ratio). It is commonly used for DQF-COSY data where resolution enhancement is necessary to prevent cancellation of nearby peaks. Another commonly used window function is the sine-squared function, which is just the square of the sine function. It rises more slowly at the beginning and drops off more gradually at the end, giving more sensitivity enhancement (when shifted 90^{o}) and better peak shape (suppression of truncation wiggles) than the sine function.

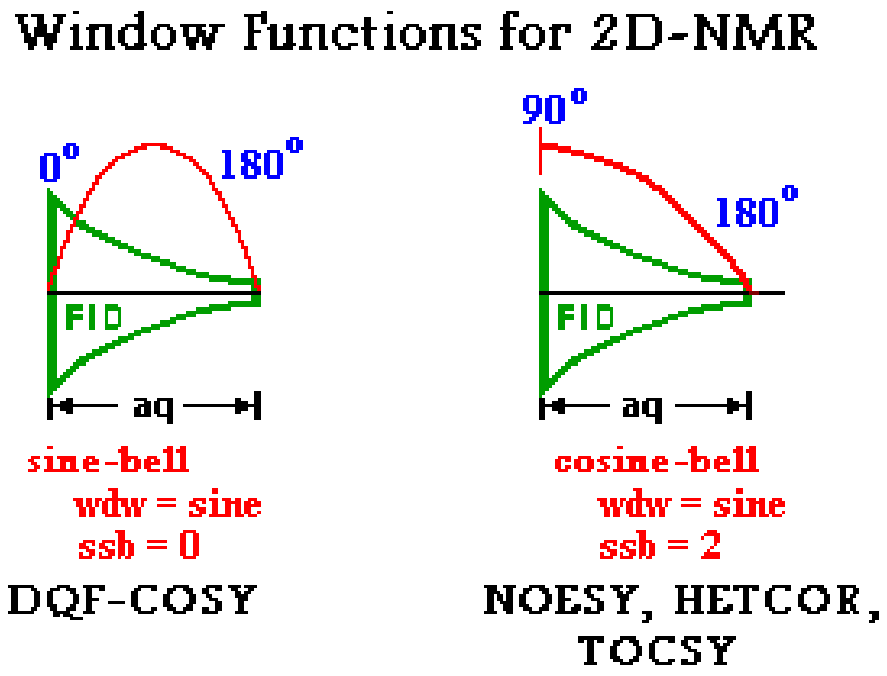

Figure 4. The two most common window functions for 2D NMR are the sine-bell, which gives resolution enhancement at the expense of sensitivity, and the cosine-bell (or 90° shifted sine-bell), which gives sensitivity enhancement with some line-broadening. Both functions come to zero smoothly at the end of the acquired FID data, eliminating the truncation wiggles in the spectrum. The Bruker parameter **wdw** determines the shape of the window function and **ssb** determines the shift.

Bruker uses the parameter **wdw** to describe the type of window function, for example **sine** for a simple sine-bell function and **qsine** for a sine-squared function. A second parameter, **ssb**, describes the amount by which the standard sine-bell (0^o to 180^o) is shifted at its starting point. If **ssb** is set to zero, the sine-bell starts at the 0^o point of the sine function. If **ssb** is set to 2, the window function starts at 90^o (180 / 2) and ends at 180^o. If **ssb** is set to 4, the window function starts at 45^o (180 / 4) of the sine function and ends at 180^o. In all cases, the function comes to zero smoothly, ending at the 180^o point of the sine function.

The units used for FIDs and spectra can be very confusing. First and foremost, all of these units are based on the data **points** by which the information is digitized. Since these points generally come in complex pairs (real, imaginary), the number of data points may refer to the total number of points (**td**) or to the number of complex pairs (**td**/2). For example, if **td** = 2048 and **si** 2048, the FID is zero-filled from 1024 (**td**/2) complex pairs to 2048 (**si**) complex pairs and then Fourier-transformed to give a real spectrum (2048 data points) and an imaginary spectrum (2048 data points). After phasing, the imaginary data points are discarded so that only the real points are displayed, resulting in a spectrum display which contains 2048 (**si**) data points. The FID can also be considered in terms of time units, or seconds. Each data point represents a time increment corresponding to the dwell time (**dw** = 1 / (2*$\mathbf{sw}(F_2)$) in F_2, **in0** = 1 / (2*$\mathbf{sw}(F_1)$) in F_1) of the acquisition, and the entire FID stretches from time zero (start of the FID) to the acquisition time (**aq** in F_2, $\mathbf{td}(F_1)$***in0** in F_1, end of the FID). After Fourier transformation, which converts from time domain to frequency domain, the units of the spectrum are either Hertz or ppm (ppm = Hz / spectrometer frequency). The width of the spectrum in ppm is always equal to the spectral width, **sw**. The zero point of the ppm (or Hz) scale of a spectrum is set by setting the reference frequency of some standard peak in the spectrum (e.g., TMS). It is important to remember, however, that underlying all of the time and frequency units is the fundamental digitization of the data, measured in data points or complex pairs of data points.

The 2D data matrix can be viewed in units of data points in both dimensions. Figure 5 shows the data matrix midway through data processing, with each t_2 FID transformed and placed into the matrix as an F_2 spectrum. The number of FIDs actually acquired in the indirect (t_1) dimension is **td** in F_1, leading to **td**(F_1)/2 complex pairs. The actual vertical size of the matrix is **si** data points which result after zero-filling from the **td**(F_1)/2 complex pairs acquired in t_1. Each peak in the F_2 spectrum results in a t_1 FID extending vertically into the acquired data for **td**(F_1)/2 data points, extended to a total size of **si** points by adding zeroes at the top of the matrix. Fourier transformation of each column of the matrix yields the completed 2D data matrix, with crosspeaks at the F_1 frequencies which were represented in the t_1 FIDs. The total number of data points in the 2D data matrix is **si**(F_2) * **si**(F_1), and since each data point requires four Bytes, the matrix occupies **si**(F_2) * **si**(F_1) * 4 Bytes of disk space. Because each Fourier transform produces a real spectrum and an imaginary spectrum, there are actually four 2D matrices saved after the double Fourier transform (**xfb** command). These are saved as files 2rr, 2ri, 2ir and 2ii (rr = real, real; ri = real, imaginary; etc.). Phase correction in two dimensions is just a process of making a linear combination of these four matrices using the phase parameters **phc0** and **phc1** in F_2 and in F_1 to generate a "real, real" matrix which has absorptive peaks in both dimensions. This is a lot of data: for a 2048 x 1024 point 2D matrix, the four matrices occupy a total of 2048 x 1024 x 4 x 4 = 33,554,432 Bytes. Because these datasets can fill up disk space very quickly, we have a "daemon" which deletes processed 2D data which is more than 3 days old. To regenerate the 2D data matrix, you only need to enter the **xfb** command.

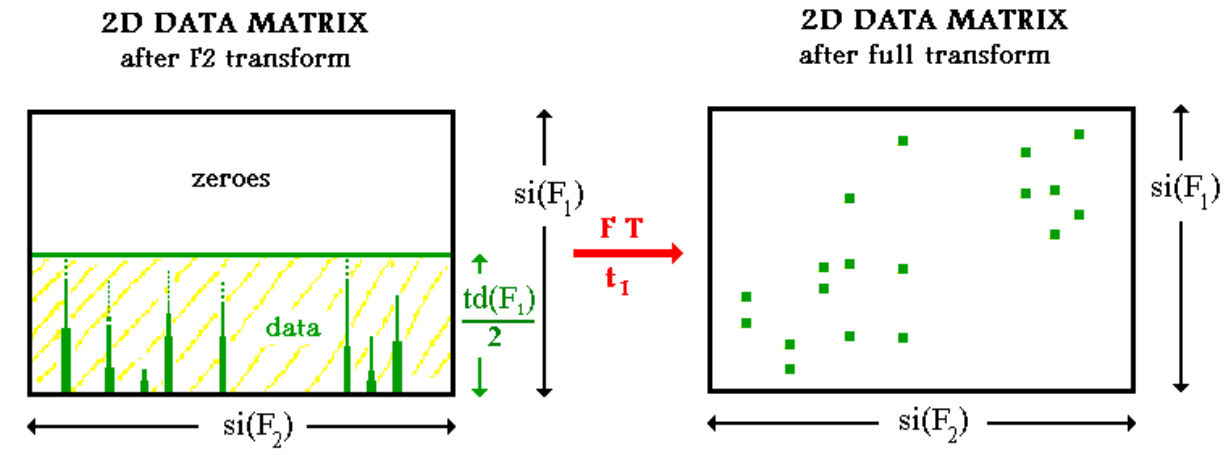

Figure 5. 2D Matrix represented in units of data points. Zero filling in t_2 increases the FID size from **td**(F_2)/2 to **si**(F_2) complex pairs, which results in a spectrum of **si**(F_2) data points after Fourier transformation and phase correction. Zero filling in t_1 extends the FID from **td**(F_1)/2 to **si**(F_1) complex pairs, which results in an F_1 spectrum of **si**(F_1) data points after Fourier transformation and phase correction. The dimensions of the final 2D spectrum in frequency units (Hz) are **sw** (F_2) in F_2 and **sw**(F_1) in F_1.

The following table summarizes the Bruker 2D parameters. In States mode (**MC2 = States**) two FIDs (real and imaginary) are acquired for each t_1 value, so that **td**(F_1) represents the number of complex pairs, or half of the number of FIDs acquired and saved. For TPPI mode (**MC2 = TPPI**), one FID is acquired for each t_1 value and **td**(F_1) represents the number of FIDs acquired and saved.

Bruker 2D Parameters

Direct (F_2)	Indirect (F_1)	
Acquisition Parameters:		
td	**td**	Number of data points to be acquired in the FID
sw	**sw**	Spectral width in ppm
dw	**in0**	Time delay between successive time samples
aq	**in0*****td**(F_1)	Acquisition time in seconds
o1p	**o2p**	Center of spectral window in ppm (heteronuclear)
o1p	**o1p**	Center of spectral window in ppm (homonuclear)
	nd0	Factor for calculating **in0** (**in0** = 1 / (**nd0*****sw**(F_1))
Processing Parameters:		
si	**si**	Number of complex pairs after zero-filling
wdw	**wdw**	Window function (sine = sin, qsine = $\sin^2$)
ssb	**ssb**	Starting point of sine-bell function = 180^o / **ssb**
phc0	**phc0**	Zero-order phase correction (chem.. shift independent)
phc1	**phc1**	First-order phase correction (chem.. shift dependent)
	mc2	Phase-encoding method for F1 (States, TPPI, etc.)